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Further analysis of a novel canine papillomavirus (CPV3) and its potential role in the context of Epidermodysplasia verruciformis

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1 Abstract

Epidermodysplasia Verruciformis (EV) is a human skin disorder characterised by widespread verrucous papulosis. EV is associated with a high risk for squamous cell carcinoma (SCC) and, aetiologically, with human Papillomaviruses (PVs). Alternatively, mutational inactivation of the anti-apoptotic p53 has been linked to SCC pathogenesis. In the present work, a dog with EV-like skin lesions was studied. Its predicted p53 sequence did not differ from the published sequence of normal dogs, indicating that p53 was not a factor in this case. However, a novel canine PV (CPV3) had been discovered in the same skin lesions. Now, samples from three different skin lesions of this dog as well as from unaffected skin were analyzed for CPV3 E6 mRNA synthesis. Indeed, CPV3 E6 mRNA was detected exclusively in the EV-like skin lesions. The putative transforming protein CPV3 E6 was expressed, as a fusion protein with the yellow fluorescent protein, from an amplicon vector in eukaryotic cells. The fusion protein accumulated in dot-like structures within the nucleus of amplicon-transduced cells and, after a few days, caused premature cell death. Thus, contrary to expectations, expression of E6 did not increase cell survival, which should be a prerequisite for cell transformation. Treatment of cells with a siRNA targeting CPV3 E6 did not result in decreased expression of the E6 protein. While CPV3 seems to be a factor in this case of EV, the underlying molecular mechanisms remain to be elucidated.

2 Introduction

2.1 Case presentation

A seven-year-old Rhodesian Ridgeback was presented at the Dermatology Unit of the Clinic for Small Animal Internal Medicine of the University of Zurich, for the evaluation of pigmented maculae and nodules and one ulcerated, interdigital plaque. The dog was affected by an expanding number of pigmented plaques slowly increasing in size. The first lesions were noticed when the dog was six months old.

Histological examination of the ulcerated plaque revealed marked acanthosis with hyperkeratosis and hypergranulosis. The epidermis was disorganized with numerous atypical cells, but the basal membrane was intact and the dermis was not affected. A diagnosis of squamous cell carcinoma (SCC) in situ was established.

Topical treatment with the immune response modifier Imiquimod was started, based on reports about successful treatment of cutaneous warts[1] and invasive SCCs[2]. Treatment did not lead to regression of the lesions. Treatment with omega interferon intralesionally and subcutaneously was not successful as well. Consequently, the affected toe had to be surgically removed.

A Papillomavirus (PV) infection was suspected and therefore a polymerase chain reaction (PCR) was performed with primers, which are able to amplify conserved regions in PVs. PV DNA was detected in different lesions.[3] To get more information on the nature of this PV, the complete viral genome was cloned. A rolling-circle amplification, which is a sequence-independent amplification of circular DNA, was performed.[4] After cloning the genome, the nucleotide sequence revealed that this PV had not been described before. It was named Canine Papillomavirus type 3 (CPV3).[5]

2.2 Causes of skin cancer

Skin cancers are primary divided into melanoma and non melanoma skin cancer, whereas the latter group includes basal and squamous cell carcinoma. In humans the pathogenesis of skin cancer is multifactorial and several causative factors have been reported. The most important cause of skin cancer is ultraviolet radiation (UVR).[6] It exerts harmful effects to the skin through direct cellular damage and alterations in immunologic function. Cellular damage occurs due to mutations in DNA, formation of thymidine dimers and alterations in p53 tumor suppressor gene. In addition immunodeficiency due to disease or due to

administration of immunosuppressive agents substantially increases the risk of skin cancer.[6] Furthermore viral infections such as the human PV can cause SCC and individuals with various familial genetic syndromes, like for example EV or Xeroderma pigmentosum, are particularly susceptible to specific types of skin cancer.[6] Besides these most important causative factors, which can be assumed to be also important factors in veterinary medicine, there are many additional risk factors like phototherapy, ionizing radiation, chemical carcinogens, aging, tanning, smoking, dermatoses, scars and diet[6], which most of them seems to be not very likely to play an important role in the pathogenesis of skin cancer in animals.

2.3 Epidermodysplasia Verruciformis

2.3.1 Relevance in humans

Epidermodysplasia Verruciformis (EV) is a rare inherited skin disease associated with a high risk of skin carcinoma that results from an abnormal susceptibility to infection by human Papillomaviruses (HPV).[7] The disease is characterized by disseminated flat warts and macular lesions, which are more or less scaly, red, brown or achromic with irregular outlines. The warts develop during early childhood and have, especially on sun exposed skin areas, a high risk to turn into SCCs in adults.[8] Furthermore an EV - like syndrome has been described, which shows clinically clearly parallels with EV, but occurs in patients with a severe immune deficiency.[9] These patients, in particular organ transplant recipients, have also a highly increased incidence of cutaneous SCCs.[10] In more than 90 % of the SCCs of EV or EV - like patients, DNA sequences specific for oncogenic EV-HPV types (predominantly HPV5 and HPV8) could be detected.[7] But EV-HPV types also occur at high frequency in normal skin of the healthy general population and in patients with benign hyperproliferative disorders.[11-14] Therefore it is difficult to determine the role of HPVs in the skin carcinogenesis.

UVR is known to be an important etiological agent in the pathogenesis of SCCs.[6,15] The facts that HPVs also occur in non affected humans and that SCCs occur predominantly at body sites exposed to UVR suggests a possible interaction between HPV and UVR in the development of SCCs in EV patients.[16] This critical dependence on UVR has been further supported by the finding of p53 mutations in over 60% of EV SCCs and up to 40 % of pre-malignant lesions.[17,18] Mutations of the p53 gene, which consequently inactivates the p53

protein occur in a wide range of human cancers. In skin cancer mutations of the p53 gene may be induced by UVR.[19]

2.3.2 Relevance in dogs

Skin lesions similar to human EV were first described in dogs by Briggs et al. and called canine lentiginosis profunda.[20] Based on its resemblance to verrucous epidermal nevus in humans this dermatosis was later designated as pigmented epidermal nevus (PEN).[21] Nagata suggested 1995 that canine PEN is equivalent to EV in humans.[22]

Affected dogs have multiple cutaneous macules, papules and plaques of heavily pigmented hyperplastic epidermis. Since this disease accumulates in certain dog breeds (pug, miniature schnauzer) it is suspected to be an inherited disorder similar to the human EV.[20,22,23] Moreover, two case reports lead to the assumption that an EV-like syndrome associated with immunsuppression occurs also in dogs: Callan et al. referred 2005 to a dog, which developed multiple epidermal harmatomas and SCCs in situ following chronic immunosuppressive therapy.[24] And Stokking et al. reported 2004 on three dogs with pigmented epidermal plaques, whereas two cases had a concurrent disease associated with impaired immune function.[25] Furthermore many publications about PV detection in such skin lesions indicate an involvement of PV in the development of the disease in dogs[22-26] and the possibility of malignant transformation of the lesions into SCC is suggested.[22,25]

2.4 p53

p53 is the most commonly mutated gene in human cancers.[27] It encodes a 53 kilodalton protein, which consists of several functional domains with specific properties. The transactivation domain is located in the N-terminal part of the protein. Sequence-specific DNA binding is mediated through the central core and the C-terminal part of p53 is composed of a flexible linker region, an oligomerization domain and a basic, regulatory C-terminal region.[27] In unstressed cells, p53 is present in a latent state and is maintained at low levels through targeted degradation.[28] In response to various stress signals, such as oncogene activation, UVR, hypoxia, virus infection and DNA damage, p53 accumulates in the nucleus and is activated as a transcription factor.[28] Activation of p53 induces or inhibits the expression of more than 150 genes, leading either to cell cycle arrest in the G1/S or G2/M phases or to apoptosis.[28] Normally, the p53 protein interacts with the Mdm2 protein,

which targets p53 for ubiquitin-mediated degradation, whereas Mdm2 is induced by p53, creating an autoregulatory loop.[27] Phosphorylation at the N-terminus reduces p53 interaction with Mdm2 with a resultant increase in p53 protein level.[27] Modification at the C- and N-termini via phosphorylation or acetylation upregulates binding to specific DNA targets increasing transcription of downstream genes, which induce cell cycle arrest, allowing time for repair of the occurred damage, or apoptosis, if the DNA damage proves to be irrepairable.[29] Loss of this protective function occurs as a consequence of p53 gene mutation, which results in a functionally impaired protein or as a consequence of p53 protein inactivation due to viral proteins such as high risk HPV E6.[29] This loss of p53 response plays an important role in tumorigenesis.[27,29]

2.5 Papillomaviruses

2.5.1 General properties

Papillomaviruses (PVs) induce warts in a variety of higher vertebrates. They are highly species specific and have a specific tropism for cutaneous or mucosal squamous epithelial cells.[30]

2.5.2 Structure and organization

The papillomavirus capsids are nonenveloped icosahedral particles about 55 nm in diameter. The viral genome consists of a single molecule of double-stranded circular DNA about 8 kilo base pairs (bp) in size.[30] About ten open reading frames (ORFs) are located on one strand of the viral DNA. These ORFs are classified as either early (E) or late (L) ORFs, based on the time of expression during infection. The E and L genes are well separated on the genome both regions ending with a poly-adenylation signal. The early region of the PV genome encodes viral proteins involved in the regulation of replication and the synthesis of viral DNA. The L1 and L2 ORFs encode the two structural capsid proteins and are expressed only in productively infected cells. Furthermore the PV genome contains a region without ORFs called long control region (LCR). This noncoding region varies slightly in size among the different PVs.[30]

2.5.3 Life cycle

The following general concept about the HPV life cycle appears to be applicable to the animal PVs that are already studied. Life cycle of HPVs is tightly connected to the differentiation of epithelial cells and depends on infection of dividing cells.[31] Basal cells in squamous epithelium can be reached by the virus due to a micro trauma in the skin. After infection, viral DNA is transported into the nucleus and genome is maintained as an episome due to expression of E1 and E2 proteins. Infected basal cells migrate towards the epithelial surface. Normally basal cells exit the cell cycle after migrating into the suprabasal cell layers and undergo a process of terminal differentiation. However this terminal differentiation is retarded by E6 and E7 proteins of PVs and the cells are kept in the proliferative phase.[31] E6 and E7 are thought to work together to achieve these effects. Whereas E7 stimulates S-phase progression and results in unscheduled S-phases, E6 prevents the induction of apoptosis, which in turn increases the chance of mutations and consequently is a predisposing factor in the development of cancer.[31] Genome amplification and packaging into infectious particles occurs in the mid or upper epithelial layers and depends of the co-expression of all viral gene products. Once viral genome amplification has been completed virus synthesis is performed in the upper layers of infected tissue by expression of the two structural proteins L1 and L2. Throughout the virus life cycle, levels of different viral proteins are controlled by promotor usage and by different splice site selection.[31]

2.5.4 Relevance in humans

A number of human papillomaviruses (HPVs) have been implicated as the etiologic agent for cervical cancer and other epithelial tumors.[30] To date, nearly hundred different HPV types have been identified based on the characterization of complete genomes, with a yet larger number presumed to exist based on the detection subgenomic amplicons.[32] The association of certain HPV types with benign and malignant lesions has led to the definition of «high-risk» types, «intermediate-risk» types and «low-risk» types.[33] About 25 HPV types preferentially infect the anogenital mucosa. In addition to their role in anogenital cancer, HPVs are also involved in the development of cutaneous lesions. These are commonly referred to as EV-HPV types and include HPV5, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47 and 49.[34]

2.5.5 HPV E6 and E7 proteins

The HPV E6 proteins are small polypeptides of approximately 150 amino acids and contain two $CX_2CX_{29}CX_2C$ motifs, which are believed to be involved in binding zinc. The E7 protein encoded by the HPVs consists of about 100 amino acids and also contains such a zinc binding motif.[30] Both proteins are consistently expressed in HPV-carrying anogenital malignant tumors and are able to immortalize a wide variety of human cell types cooperatively.[35] In case of high risk anogenital HPV types, the viral transforming genes E6 and E7 are therefore thought to be responsible for the development of cancer.[36] The E6 protein initiates degradation of the cellular tumor suppressor protein p53 via an ubiquitin-mediated pathway.[37] The p53 protein plays a key role in cellular responses to stress factors, such as DNA damage or hypoxia, principally through an induction of cell cycle arrest or apoptosis.[38] Furthermore the E6 protein targets a variety of other host-cell proteins, for example it mediates the degradation of the pro-apoptotic protein, Bak, a member of the Bcl-2 family.[35] Similar to E6, the E7 protein functions are also pleiotropic. Amongst others, it binds to the cellular tumor suppressor protein pRB and to the retinoblastoma protein-related pocket proteins, what results in enhanced degradation of these proteins.[35,39]

In contrast to high risk anogenital HPV types, the E6 protein of EV HPV types is unable to promote p53 degradation.[40,41,42] But E6 proteins of different cutaneous HPVs have been shown to inhibit p53-dependent and p53-independent apoptotic pathways in response to UVR damage.[16] Jackson et al. showed 2000 that the expression of the pro-apoptotic protein Bak increases in human kerationcytes treated with UVR, but not in cell lines expressing the cutaneous HPV E6 proteins. They suggested that E6 proteins of cutaneous HPVs promote proteolytic degradation of Bak and that this elimination of Bak protein leads to a decrease in apoptosis in UV-irradiated cells.[43]

2.5.6 Relevance in dogs

To date only canine oral PV (COPV) has been studied extensively. It induces benign papillomas in the mucous membrane of the oral cavity of dogs. The nature and the biological properties of the remaining canine PVs is largely unknown. Recently Zaugg et al. reported the detection of novel PVs in canine SCCs and suggested that those PV contribute to the development of malignancy and that the differentiation between low and high risk canine PV is feasible.[3]

2.5.7 Canine Papillomavirus 3

The CPV3 genome counts 7801 bp and has a typical PV genome structure with an early region (E1, E2, E6, and E7), a late region (L1, L2) and a non-coding LCR between the end of L1 and the beginning of E6.[5] The genomic map of CPV3 DNA is shown in Figure 1. The size and the content of $CX_2CX_{29}CX_2C$ motifs of CPV3 E6 and E7 proteins are similar to HPV E6 and E7 proteins. The sequence of CPV3 is 56% and 55% identical to COPV or CPV2 on the nucleotide level.[5] Unlike COPV, whose genome contains a large non coding sequence between the E2 and L2 ORF (1500 bp), the CPV3 gap between these two ORFs is only 211 bp. Phylogenetic analysis, based on the nucleotide sequence coding for the L1 protein, does not group CPV3 into one of the defined PV genera.[5]

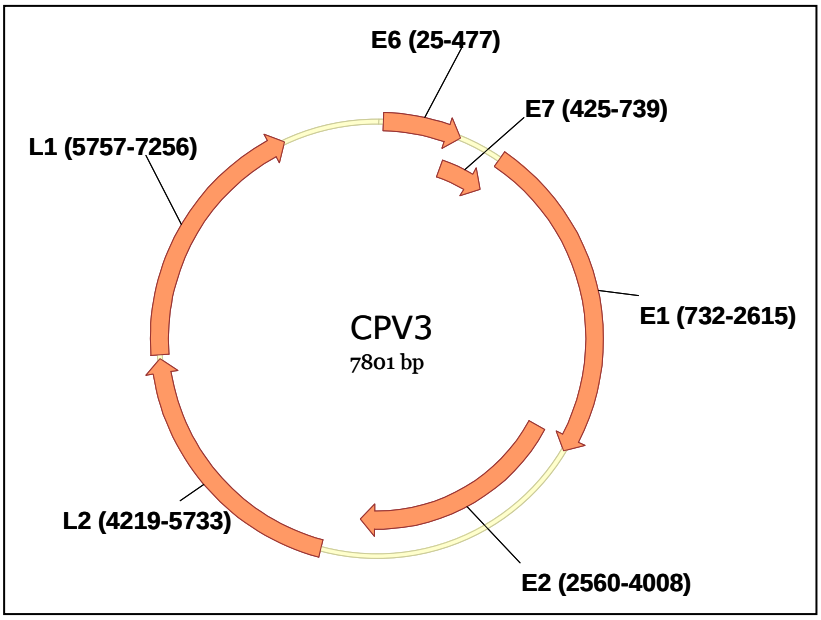


Figure 1: CPV3 genomic map. The numbers show the nucleotide positions of the ORFs.

2.6 Herpesvirus amplicon system

Spaete and Frenkel detected 1982 that an origin of DNA replication (ori) and a packaging signal (pac) are the only two cis-acting sequences required for replication and packaging of defective virus genomes in the presence of a trans-acting herpes simplex virus-1 (HSV-1) helper-virus.[44] This primary observation was adapted to develop the HSV-1 amplicon system. The amplicon system is a versatile vector for heterologous expression of genes *in*

vitro and *in vivo*. An amplicon vector containing a transgene cassette with the gene of interest, sequence elements of HSV-1, including an ori- and a pac-signal is cotransfected together with a cloned HSV-1 helper virus genome lacking the pac-signal into permissive cells. This results in virions filled with amplicon DNA which can subsequently be used for transduction of cells in order to express the gene of interest.[44] Heterologous expression of genes using the amplicon system has two main advantages over the transfection of plasmid DNA. First, the expression is tunable in a sense that the more amplicons are applied to cells the more cells express the gene of interest and second, transduction is better tolerated by cells than transfection with lipid/DNA complexes.[44]

2.7 RNA interference

RNA interference is a sequence specific, posttranscriptional gene silencing mechanism, which is triggered by small interfering, double-stranded (ds) RNA (siRNA) and causes degradation of mRNA homologous in sequence to the siRNA.[45] siRNAs consist of about 21 nucleotides and are naturally produced as a part of the RNA interference pathway, which has been shown to knock down a large number of genes expressed in mammalian cells.[45] siRNAs are generated when long ds RNAs are recognized and digested by an RNAse-like enzyme called Dicer. Afterwards they are incorporated into a multicomponent nuclease called RISC (RNA induced silencing complex) that mediates unwinding of the siRNA duplex and targeting of mRNA. This binding mediates target mRNA to be cleaved by Dicer. The cleaved mRNA can be recognized by the cells as being aberrant and be destroyed.[45] The process of RNA interference is self-replicative, while siRNAs simultaneous serve as primers to transform the target mRNA into more ds siRNA molecules.[46] Such siRNA targetting specific mRNA can exogenously introduced for specific inhibition of gene expression.[47,48] Butz et al. showed 2003 that siRNAs directed against HPV E6 provide very efficient molecular tools to inhibit intracellular E6 activities specifically.[49]

2.8 Aim of the study

The aim of this study was to further analyse the role of CPV3 in the development of the skin lesions of the affected Rhodesian Ridgeback. Other causative factors in the pathogenesis of SCCs have been investigated and were excluded. No evidence of immunosupression was found in the history of the dog. A congenital disorder was not suspected since no relatives of the dog showed a similar skin disease. However there is no possibility to exclude this option. The possibility of an UVR induced mutation of the p53 gene was eliminated by determination of the mRNA nucleotide sequence of this gene. No other causative factor for the SCC development could be identified. Thus the CPV3 infection became more important. Therefore, our next step was to determine viral gene expression by evaluating the presence of CPV3-E6 mRNA in skin lesions of the affected dog. Detection of CPV3-E6 mRNA in three different skin lesions, but not in healthy skin, underlined the importance of the CPV3 infection in skin cancerogenesis. To further evaluate the involvement of PVs in the development of skin lesions, it was necessary to investigate the role of the putative transforming proteins E6 and E7, which may be crucial for cancerogenesis. Therefore the next step was to characterize the CPV3 E6 protein in two ways: First, by observation of the expression and localization of the CPV3-E6 protein in eukaryotic cells. And second, by examination of the effects of a siRNA targeting E6 on the expression of CPV3 E6 in cell cultures.

3 Material and Methods

3.1 Sample Collection

Skin samples were taken whilst the dog was conscious. 0,5ml of 1% Lidocain was infiltrated into the subcutis under the sites marked for biopsy. One non-lesional and one lesional sample was taken from the abdomen. The other two lesional samples were taken from the thorax. Six-millimetre diameter skin biopsies were taken, cut into two pieces, placed immediately into liquid nitrogen and then stored at –80℃.

3.2 Plasmids

3.2.1 pTOPO-p53

pTOPO-p53 was constructed by using the TOPO TA Cloning Kit (Invitrogen, Basel, Switzerland). For amplification of the p53 transcripts of the affected dog RNA of healthy skin and of lesion 3 was extracted, reverse transcribed as described below (3.3.1) and subsequently amplified by using the p53+2 and p53neu- primers (Table 1). PCR reaction was performed in the thermocycler PTC 200. The following reagents were added in a 200µl PCR-reaction tube: 1µl dNTP (10mM), 2µl of each primer (10µM), 5µl cDNA, 10µl 5X Phusion High-Fidelity buffer and 0,5µl (1unit) Phusion High-Fidelity DNA-Polymerase (Finnzymes, Espoo, Finland). The mix was topped up to 50µl with ddH_2O. PCR conditions involved an initial denaturation step at 98℃ for 2 min, followed by 35 cycles consisting of a denaturation step at 98℃ for 10sec, an annealing s tep at 52℃ for 30sec and an extension step at 72℃ for 45sec, and by a final extension st ep at 72℃ for 10min. The PCR product was run through an agarose gel and cleaned by using the QIAEX II Agarose Gel Extraction Kit (Qiagen, Basel, Switzerland). 20µl of the eluted DNA were incubated with 0,1µl Taq DNA Polymerase and 1µl 10 mM dATP`s for 15min at 72℃ to create 3´ overhanging As and cloned into the pCR 2.1-TOPO vector according the manufacturers instructions (Invitrogen Basel, Switzerland). In brief, the cloning reaction consisted of 4µl PCR product, 10ng vector and 1µl salt solution. The reaction was gently mixed and incubated on ice for 5min. Afterwards, 2µl of the reaction were transformed into chemically competent TOP 10 *E.coli* (Invitrogen, Basel, Switzerland) and plated onto LB-Agar plates containing 100µg/ml ampicillin. Colonies were expanded over night at 37℃ in LB supplemented with 100µg/ml ampicillin and plasmid DNA was extracted with the Sigma

GenElute™ HP Plasmid Purification Kit (Sigma-Aldrich Corporation, Switzerland, Buchs). The sequences were determined by Microsynth AG Sequencing Group (Balgach, Switzerland).

3.2.2 pHSV-E6-EYFP

To construct a Herpesvirus amplicon vector the Gateway technology (Invitrogen, Basel, Switzerland) was used. A coding region of CPV3 E6 was obtained by PCR amplification of pCPV3 with the appropriate primers (E6++ and E6-, Table 1). PCR reaction was performed in the thermocycler PTC 200. The following reagents were added in a 200µl PCR-reaction tube: 2µl dNTP (10mM), 2,5µl of each primer (10µM), 1µl plasmid DNA (provided by the Institute of Virology, Zurich, Switzerland), 5µl 10X Pfu reaction buffer and 1µl (2,5units) PfuTurbo DNA polymerase (Stratagene, Amsterdam, The Netherlands). The mix was topped up to 50µl with ddH_2O. PCR conditions involved an initial denaturation step at 95°C for 2min, followed by 35 cycles consisting of a denaturation step at 95°C for 1min, an annealing step at 47°C for 1min and an extension step at 72°C for 1min, and by a final extension step at 72°C for 10min. The PCR product was cleaned by using the QIAEX II Agarose Gel Extraction Kit (Qiagen, Basel, Switzerland) and directionally cloned into the pENTR/D-TOPO vector. The CACC sequence at the 5`end of the forward primer paired with the overhang sequence GTGG in the pENTR/D-TOPO vector and enabled the directional cloning of the blunt end PCR products into an entry clone. The cloning reaction consisted of 23.2ng PCR product, 20ng TOPO vector, 1µl salt solution and water added to a final volume of 6µl. The reaction was gently mixed and incubated for 5min at room temperature (RT). Afterwards, 2µl of the reaction were transformed into competent TOP 10 *E.coli* (Invitrogen, Basel, Switzerland) and plated onto LB-Agar plates containing 50µg/ml kanamycin. After sequencing of the resulting clone (pENTR-E6) the LR recombination reaction between this entry clone and the destination vector pHSV-EYFP-RfC (provided by the Institute of Virology, Zurich, Switzerland) was performed. The recombination reaction consisted of 254ng entry clone, 425ng destination vector, 4µl 5x LR clonase buffer and TE buffer pH 8.0 added to a final volume of 16µl. After gently mixing the reaction, 4µl of LR Clonase enzyme mix were added, the reaction mix was mixed well and incubated at 25°C for 1 hour. After adding of 2µl of Proteinase K solution the reaction mix was incubated for 10min at 37°C. 1µl of the recombination reaction was transformed into competent TOP 10 *E.coli* (Invitrogen, Basel, Switzerland) and plated onto LB-Agar plates containing 100µg/ml ampicillin. For purification of plasmid DNA the Sigma GenElute™ HP Plasmid Purification

Kit (Sigma-Aldrich Corporation, Switzerland, Buchs) was used. Clones were checked by restriction enzyme analysis and sequence analysis was done by Microsynth AG Sequencing Group (Balgach, Switzerland).

3.2.3 pTOPO-GAPDH

pTOPO-GAPDH was constructed by using the TOPO TA Cloning Kit (Invitrogen, Basel, Switzerland). For amplification of canine GAPDH RNA of canine skin was extracted and reverse transcribed as described later (3.3.1) and subsequently amplified by using the GAPDH2+ and GAPDH2- primers (Table 1). PCR conditions involved an initial denaturation step at 94℃ for 3min, followed by 35 cycles consisting of a denaturation step at 94℃ for 30sec, an annealing step at 55℃ for 30 sec and an extension step at 72℃ for 30sec, and by a final extension step at 72℃ for 5m in. The PCR product was run through an agarose gel and cleaned by using the QIAEX II Agarose Gel Extraction Kit (Qiagen, Basel, Switzerland) and directionally cloned into the pCR 2.1-TOPO vector. The cloning reaction consisted of 82ng PCR product, 10ng vector, 1µl salt solution and water added to a final volume of 6µl. The reaction was gently mixed and incubated on ice for 5min. Afterwards, 2µl of the reaction were transformed into competent TOP 10 *E.coli* (Invitrogen, Basel, Switzerland) and plated onto LB-Agar plates containing 25µg/ml kanamycin. Plasmid DNA purification was done with the Sigma GenElute™ HP Plasmid Purification Kit (Sigma-Aldrich Corporation, Switzerland, Buchs) and sequence analysis (Microsynth AG Sequencing Group, Balgach, Switzerland) confirmed that pTOPO-GAPDH contained the desired GAPDH insert.

Primer Target	Method	Term	Sequence
CPV3-E6	PCR	E6++	5`-CACCATGGAGCGTCCATGGAGC-3`
CPV3-E6	PCR	E6-	5`-AGAACGATGTCTCTCAAAGTAGCG-3`
CPV3-E6	Real Time PCR	TaqE6+	5`-TCCAAGCACTCTACAGAAGACAG-3`
CPV3-E6	Real Time PCR	TaqE6-	5`-AGCGTCTTTCCCAATCATTATCAG-3`
Canine GAPDH	PCR and Real Time PCR	GAPDH2+	5`-CGGCATCGTGGAGGGCCTCATGAC-3`
Canine GAPDH	PCR and Real Time PCR	GAPDH2-	5`-ATGCCAGTGAGCTTCCCGTTCAGC-3`
Canine p53	Reverse Transcription	p53-	5`-TTAACCTCAGTCTGAGTCAAGCCCT-3`
Canine p53	PCR	p53+2	5`-ATGCAAGAGCCACAGTCAGAGCTC-3`
Canine p53	PCR	p53neu-	5`-GATTGCCCCTTCTTTGCCTTCA-3`

Table 1: Primers for amplification of CPV3 E6, canine GAPDH and canine p53

3.2.4 pHSV-E6-mRFP

To replace the yellow fluorescent protein gene of pHSV-E6-EYFP with the red fluorescent protein gene of pcDNA-mRFP1-N (provided by the Institute of Virology, Zurich, Switzerland), the plasmid DNAs were digested with Asp718 and *Eco*RI and subsequently analysed on agarose gel. The desired DNA fragments were cleaned using the QIAEX II Agarose Gel Extraction Kit (Qiagen, Basel, Switzerland) and ligated. The ligation reaction mix consisted of 120ng of linearized pHSV-E6, 55ng of excised mRFP, 1.2µl ligase buffer, 1µl ligase and 4.3µl water and was incubated at 25°C for 30min. Afterwards, 4µl of the ligation reaction were transformed into competent TOP 10 *E.coli* (Invitrogen, Basel, Switzerland) and plated onto LB-Agar plates containing 100µg/ml ampicillin.

3.2.5 pHSVsiE6

Amplicon vector pHSVsuper (provided by the Institute of Virology, Zurich, Switzerland) contains an HSV-1 origin of DNA replication (OriS), a HSV-1 DNA packaging signal (Pac), EGFP under control of the HSV-1 IE 4/5 promotor, a RNA polymerase III-dependent H1 promotor and a well-defined start of transcription and a termination signal. This vector was digested with *Bgl*II and *Hind*III, analysed on agarose gel and the desired DNA fragment was cleaned using the QIAEX II Agarose Gel Extraction Kit (Qiagen, Basel, Switzerland). DNA oligonucleotides targeting CPV3 E6 at two different locations (Table 2) were synthesized (Microsynth, Balgach, Switzerland) as 64-mer sense and antisense

oligonucleotide templates (2 x 19 nucleotides specific to the targeted sequences and 26 nucleotides for restriction enzyme recognition sites and hairpin structure) as described previously.[58] These oligonucleotides were annealed and ligated with the linearized pHSVsuper. The annealing reaction mix consisted of each 3µg sense and antisense oligonucleotide template and 48µl annealing buffer (50mM HEPES pH 7.4, 25 mM NaCl) and was incubated at 95℃ for 5min, at 70℃ for 10m in and then slowly cooled down to 4℃. The ligation reaction mix consisted of 2µl of the annealed oligonucleotides, 2µl of digested pHSVsuper, 1µl ligase buffer, 1µl ligase and 4µl water and was incubated at 14℃ over night (o/n). Afterwards, 2µl of the ligation reaction were transformed into MAX Efficiency DH10B™ competent cells (Invitrogen, Basel, Switzerland) and plated onto LB-Agar plates containing 100µg/ml ampicillin. The resulting amplicons were designated pHSVsiE6-2 and pHSVsiE6-3.

siRNA	Target nt	Sequence
CPV3 E6-2	4035-4053	AGAGAGGTGTCACAGTACA
CPV3 E6-3	5013-5031	ATACCAGTGAGGTGCACAT

Table 2: siRNA target sequences within the CPV3 E6 ORF

3.3 Real time PCR

3.3.1 RNA Extraction and Reverse Transcription

Total RNA was extracted from the skin biopsies by using the RNeasy Mini Kit with RNase-Free DNase Set (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. The extracted RNA was eluted in 40µl RNase free water and divided into 5 aliquots. Each aliquot of RNA was again treated with 1µl DNase (Promega, Madison, WI) to degrade the residual DNA. After an incubation of 30min at 37℃, 1µl DNase Stop solution was added and the reaction was incubated at 75℃ to destroy D Nase. Reverse transcription was done with the Reverse Transcription System (Promega, Madison, WI) by using oligo dT primer to reverse transcribe mRNA. The reverse transcription reaction contained 4µl MgCl, 2µl RT buffer, 2µl dNTPs, 0.5µl RNasin, 1µl Oligo dT primer, 0.75µl reverse transcriptase and one aliquot of RNA. As a negative control another aliquot of RNA was mixed in exactly the same way but instead of 0.75µl reverse transcriptase 0.75µl water was added (=no-RT

mRNA sample). For the generation of p53 cDNA 1µl of a 10µM gene-specific reverse primer (p53-) was used instead of the Oligo dT primer.

3.3.2 Quantitative PCR analysis of skin samples

Real-time quantitative PCR was performed in single wells of a 96-well plate in a 25µl reaction volume using the iCycler IQ^{TM} (Bio-Rad, Hercules, CA). The 25µl reaction mixture contained 12.5µl IQ^{TM} SYBR Green Supermix (2X) (Bio-Rad, Hercules, CA), 5pmol of forward and reverse primer, 10µl of different dilutions of cDNA and water was added up to 25µl. The used primers are listed in Table 1 (TaqE6+, TaqE6-, GAPDH2+ and GAPDH2-). Reactions were amplified in duplicate and incubated according to the iCycler 2StepAmp+Melt.tmo protocol (BioRad, Hercules, CA). The corresponding no-RT mRNA sample was included as a negative control and one sample consisting of distilled water was amplified to control contamination. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Data were analyzed with the iCycler iQ Real-Time PCR Detection System Software, version 3.1, Biorad. The conditions for the determination of the Ct-values were standardised as follows: baseline cycles were defined from cycle 2 through cycle 19 and the threshold position was placed at 50 fluorescent units. Samples were considered negative if the Ct values exceeded 40 cycles.

3.4 Cells

VERO cells and VERO 2-2 cells were maintained in Dulbecco`s modified Eagle medium (DMEM) (Gibco BRL, Life Technologies, Basel, Switzerland) supplemented with 2%, 6% or 10% fetal calf serum (FCS) (AMIMED, BioConcept, Allschwil, Switzerland), 100 units/ ml Penicillin G (Sigma, Buchs, Switzerland) and 75 units/ ml Streptomycin (Sigma, Buchs, Switzerland) in a humidified 5% CO_2 incubator at 37℃. To prevent contamination, all w ork was done in the laminar flow. Pictures of the cells were taken using an inverse microscope Zeiss Axiovert S100 with or without appropriate UV-filter.

3.4.1 Transfection of VERO cells with pHSV-E6-EYFP

For transfection of VERO cells with the pHSV-E6-EYFP amplicon vector a 6 well plate containing 4x10^5 VERO cells per well was incubated o/n. For each dish, 100µl OptiMEM were mixed with 1.6µg pHSV-E6-EYFP DNA. In a second tube 100µl OptiMEM were mixed with 4µl LipofectAMINE (Invitrogen, Basel, Switzerland). Both tubes were incubated

for 5min at RT. After gently mixing both tubes, the content of the tubes were combined, mixed and incubated for 20min at RT. Afterwards 1ml OptiMEM was added and the transfection mix was applied to the cells which were then incubated for 5 hours at 37℃. Thereafter, the transfection mix was aspirated from the cells and they were washed with DMEM (0% FCS). Finally, 2ml DMEM (2% FCS) were added and the cells were incubated for 48 hours. To visualize the nuclei cells were stained with 0.01µg/ ml Hoechst 33342 (Invitrogen, Basel, Switzerland).

3.4.2 Packaging of pHSV-E6-EYFP into HSV-1 particles

For packaging of pHSV-E6-EYFP into HSV-1 particles 4 x 6cm^2 tissue culture dishes containing 1.2x10^6 VERO 2-2 cells were incubated over night. For each dish, 250µl OptiMEM were mixed with 2µg fHSVΔpacΔ27 DNA, 0.2µg pEBHICP27 DNA and 0.4µg pHSV-E6-EYFP DNA. After overlaying with 10µl Plus reagent the reaction mix was incubated for 10min at RT. Thereafter it was carefully mixed and incubated for another 5min at RT. In a second tube 250µl OptiMEM were mixed with 16µl LipofectAMINE (Invitrogen, Basel, Switzerland) and incubated for 5min at RT. After gently mixing of both tubes, the content of the tubes were combined and incubated for 30min at RT. Afterwards 900µl OptiMEM were added and the transfection mix was applied to the cells which were incubated for 4 hours at 37℃. Thereafter, the tran sfection mix was aspirated from the cells and they were washed with OptiMEM. Finally 3.5ml DMEM (6% FCS) were added and the cells were incubated for three days. To harvest the amplicon particles the VERO 2-2 were scraped into the medium 3 days post co-transfection. The cell suspension was sonicated on ice for 20sec with 20% output energy and the cell debris was removed by centrifugation. The supernatant was divided into aliquots and these were frozen in an ethanol-dry ice bath. The frozen aliquots were stored at –70℃.

3.4.3 Transduction of VERO/ VERO 2-2 cells with pHSV-E6-EYFP amplicon particles

For transduction of VERO/ VERO 2-2 cells with pHSV-E6-EYFP amplicon particles a 12 well plate containing 1,8x10^5 cells per well was incubated over night. Next day 200µl of the amplicon particles were added to the cells. After incubation for four hours the cells were washed with PBS, 1ml DMEM (2% FCS) was added and the cells were incubated for 24 hours.

3.4.4 Packaging of pHSVsiE6-2, pHSVsiE6-3, pHSVsiEGFP and pHSVsuper into HSV-1 particles

For packaging of the siRNAs pHSVsiE6-2 and pHSVsiE6-3 and the controls pHSVsiEGFP and pHSVsuper (provided by the Institute of Virology, Zurich, Switzerland) into HSV-1 particles 6 x 6cm^2 tissue culture dishes containing 1.2x10^6 VERO 2-2 cells were incubated over night. For each dish, 250µl OptiMEM were mixed with 2µg fHSVΔpacΔ27 DNA, 0.2µg pEBHICP27 DNA and 0.4µg pHSV-E6-EYFP DNA. After overlaying with 10µl Plus reagent the reaction mix was incubated for 10min at RT. Thereafter it was carefully mixed and incubated for another 5min at RT. In a second tube 250µl OptiMEM were mixed with 16µl LipofectAMINE (Invitrogen, Basel, Switzerland) and incubated for 5min at RT. After gently mixing of both tubes, the content of the tubes were combined and incubated for 30min at RT. Afterwards 900µl OptiMEM were added and the transfection mix was applied to the cells which were incubated for 4 hours at 37℃. Thereafter, the transfection mix was aspirated from the cells and they were washed with OptiMEM. Finally 3.5ml DMEM (6% FCS) were added and the cells were incubated for three days. To harvest the infectious amplicon particles the VERO 2-2 were scraped into the medium 3 days post co-transfection. The cell suspension was sonicated on ice for 20sec with 20% output energy and the cell debris were removed by centrifugation. The supernatant was purified and concentrated using a sucrose gradient. Afterwards the amplicon particles were resuspended o/n in 280µl HBSS at 4℃. On the next day, the amplicon-HBSS mix was gently mixed, divided into aliquots, which were frozen in an ethanol-dry ice bath. The frozen aliquots were stored at –70℃. Titration was done by counting the number of green cells 24 h following infection of VERO 2-2 cells using a fluorescence microscope.

3.4.5 Transduction of VERO 2-2 cells with siRNA amplicon particles and control amplicon particles

For transduction of VERO 2-2 cells with pHSVsiE6-3, pHSVsiEGFP and pHSVsuper amplicon particles a 24 well plate containing 5x10^4 cells per well was incubated o/n. Next day 1x10^5 transducing units (TU) were mixed with 250µl DMEM (2% FCS) and added to the cells. After incubation for four hours the cells were washed with PBS, 0.5ml DMEM (2% FCS) was added and the cells were incubated for 24 hours.

3.4.6 Transfection of VERO 2-2 cells with pHSVE6-mRFP

To transfect the transduced VERO 2-2 cells with pHSV-E6-mRFP, for each well 25µl OptiMEM, 0.25µg pHSV-E6-mRFP DNA and 4µl PLUS reagent (Invitrogen, Basel, Switzerland) were mixed and incubated at RT for 15min. In a second tube 25µl OptiMEM were mixed with 1µl LipofectAMINE (Invitrogen, Basel, Switzerland). After gently mixing both tubes, the content of the tubes were combined, mixed and incubated for 15min at RT. Afterwards 250µl OptiMEM was applied and the transfection mix was added to the cells which were incubated for 4 hours at 37℃. Thereafter, the transfection mix was aspirated from the cells and they were washed with DMEM (0% FCS). Finally, 0.5ml DMEM (2% FCS) was added and the cells were incubated for 24 hours.

4 Results

4.1 Sequencing of p53

In order to include or exclude a p53 gene mutation as a causative factor in the development of skin lesions, RNA of healthy skin and of lesion 3 was extracted, reverse transcribed with a gene specific reverse primer, amplified by PCR and cloned into pCR 2.1-TOPO as described in Material and Methods. Agarose gel electrophoresis of the PCR products revealed a band of the expected size (1091 bp) corresponding to a large fragment of the p53 ORF (Figure 5). The isolated and purified PCR products were used for the TOPO cloning reaction. Whereas the resulting plasmid pTOPO-p53_1 contained the p53 molecule of the healthy skin and plasmid pTOPO-p53_2 contained the p53 molecule of lesion 3. To identify clones containing the desired piece of DNA, ten colonies of each construct were selected, plasmid DNA prepared and restriction analysis with two restriction enzymes (*Sac*I and *EcoR*I) was performed. For both constructs six of the ten isolated clones showed the expected restriction pattern. To analyze the p53 sequence, two clones of each construct showing the calculated restriction pattern were sequenced (pTOPO-p53_1D, pTOPO-p53_1F, pTOPO-p53_2C and pTOPO-p53_2G). Sequence analysis revealed that the inserted p53 molecule of every analysed clone contained the same nucleotide sequence. Setoguchi et al. reported 2001 about the canine wild type p53 sequence, consisting of 1247 bp, which contained the 5´noncoding region (46 bp), the ORF (1146 bp) and the 3`noncoding region (55 bp). Comparison of our 1091 bp long sequence (covering nearly the entire p53 ORF) with the 1247 bp long wild type revealed, that these 1091 bases were completely identical.[50] The shortening of the cloned p53 fragment resulted from the method used to obtain the amplified cDNA (reverse transcription with specific primer followed by PCR with a nested reverse primer). Primers for PCR amplification reached from nucleotide 47 to 70 and from nucleotide 1137 to 1116. Therefore we effectively analysed 1045 nucleotides, corresponding to codon 29 - 356. These results suggest that the analysed p53 ORF fragment did not show any mutation, neither in healthy skin nor in lesion 3.

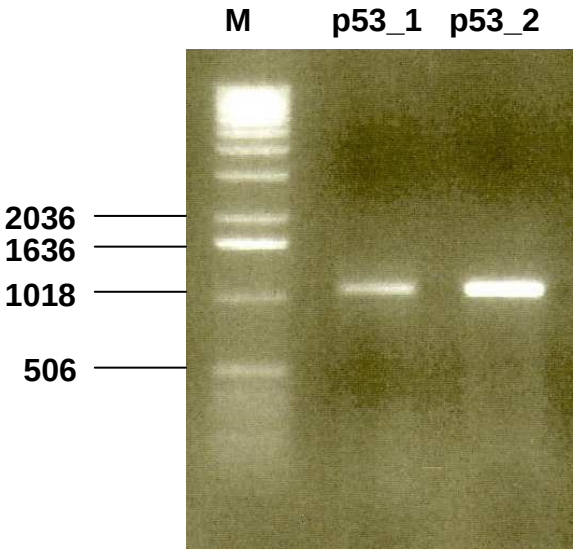


Figure 5: Agarose gel analysis of the PCR product
M: 1kb DNA ladder; p53_1: healthy skin, p53_2: lesion 3

4.2 Cloning of CPV3 E6 into an amplicon destination vector

In order to construct a Herpesvirus amplicon vector containing the CPV3 E6 open reading frame (ORF), the sequence corresponding to the E6 ORF was amplified by PCR and cloned into pENTR/D-TOPO vector as described in material and methods.

Agarose gel analysis of the PCR product revealed a band of the expected size (450 bp) corresponding to the E6 ORF (Figure 2a). The isolated and purified PCR product was used for the TOPO cloning reaction. To identify clones containing the desired E6 ORF, 10 colonies were picked, plasmid DNA isolated and restriction analysis with two restriction enzymes (*Ava*I and *Mlu*I) was performed. Five of the ten isolated clones showed the calculated restriction pattern. Figure 2b shows the agarose gel analysis of the five positive clones. To confirm that the E6 ORF was cloned in the proper orientation and contained the correct nucleic acid sequence, two clones showing the calculated restriction pattern were sequenced (pENTR-E6_B and pENTR-E6_C). By performing a Gateway LR recombination reaction between the pENTR-E6_B and an amplicon destination vector, containing the EYFP gene in frame with the recombination cassette RFC C.1 (pHSV-EYFP-RfC), an expression clone containing the desired E6 ORF was generated (pHSV-E6-EYFP). The resulting recombination reaction was again transformed into E.coli. Plasmid DNA was isolated from 6 colonies and restriction analysis with three restriction enzymes (*Not*I, *Apo*I and *Blp*I) was performed to confirm the presence of the insert. All the isolated expression clones showed the calculated restriction pattern. Figure 2c shows the

restriction pattern of two expression clones (pHSV-E6-EYFP_K and pHSV-E6-EYFP_L). Sequence analysis confirmed that these two expression clones contained the E6 ORF in frame with EYFP. These results suggest that an amplicon expression clone containing the E6 ORF in frame with EYFP had been generated.

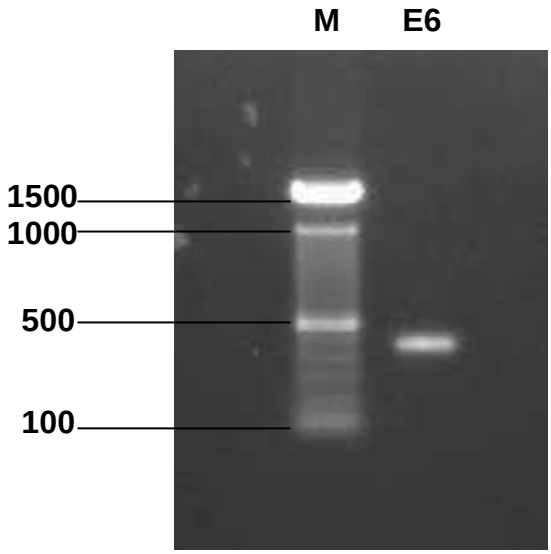


Figure 2a: Agarose gel analysis of the PCR product
M: 100bp DNA ladder; E6: PCR product

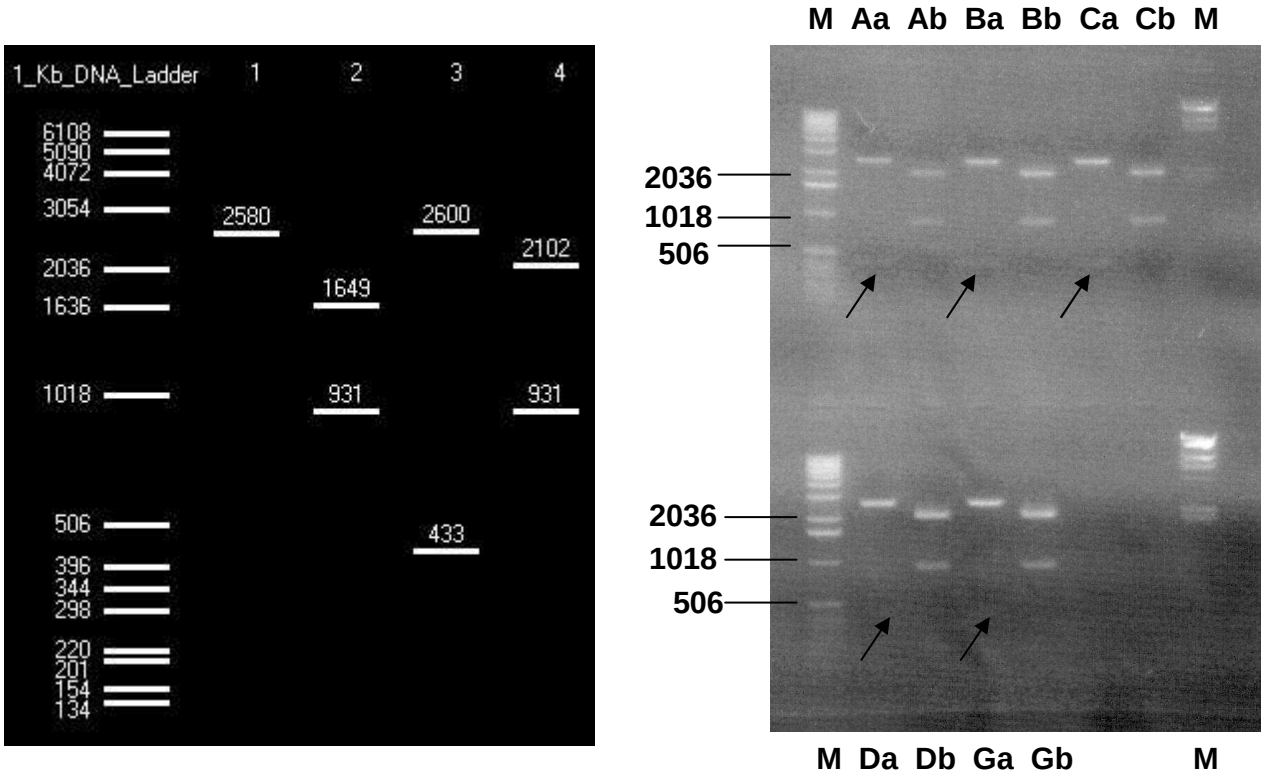


Figure 2b: Restriction analysis of the entry clone pENTR-E6 on agarose gel
Left panel: virtual digestion:
Lane 1: *Ava*I digestion of pENTR/D-TOPO: calculated fragment: 2580bp
Lane 2: *Mlu*I digestion of pENTR/D-TOPO: calculated fragments: 1649bp, 931bp
Lane 3: AvaI digestion of pENTR-E6: calculated fragments: 2600bp, 433bp
Lane 4: *Mlu*I digestion of pENTR-E6: calculated fragments: 2102bp, 931bp
Right panel: digestion of pENTR-E6:
M: 1kb DNA ladder; A, B, C, D, G: clone A, B, C, D, G; a: *Ava*I digestion; b: *Mlu*I digestion.
The arrow indicates the position of the 433bp fragment that was only faintly visible

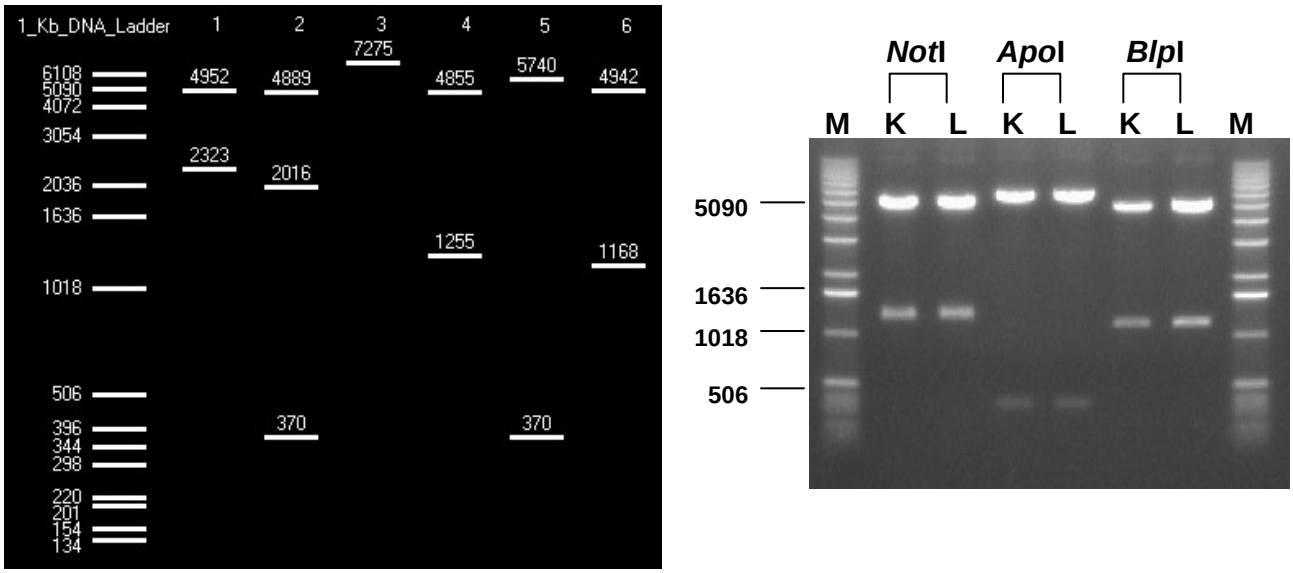


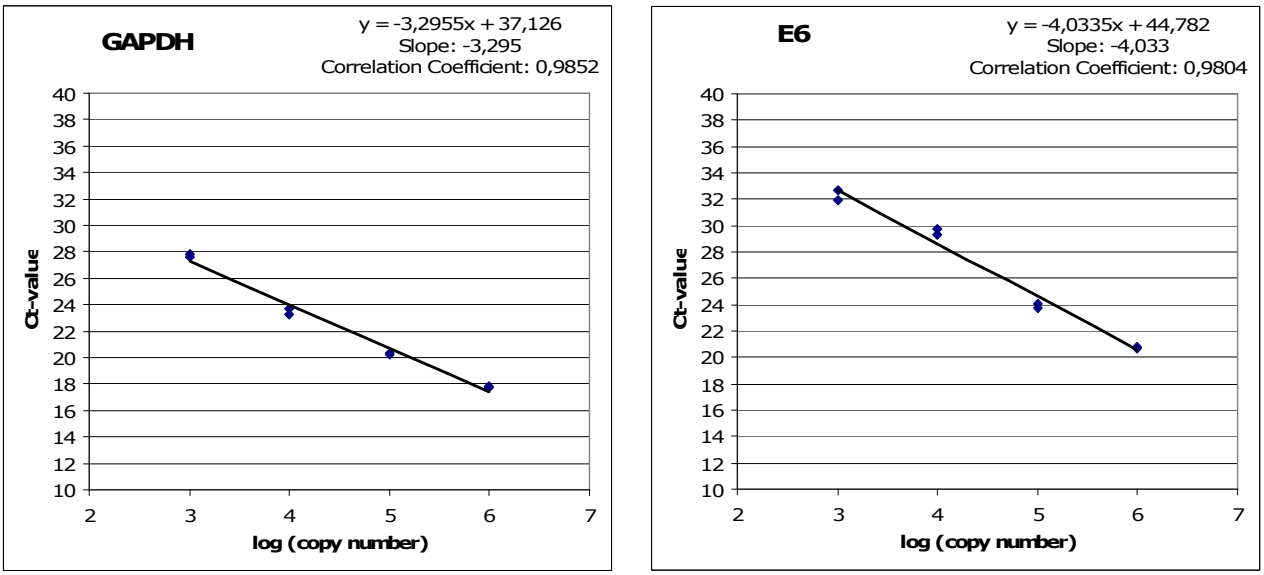
Figure 2c: Restriction analysis of the amplicon expression clone pENTR-E6-EYFP on agarose gel
Left panel: virtual digestion:
Lane 1: *Not*I digestion of pHSV-EYFP-RfC: calculated fragments: 4952bp, 2323bp
Lane 2: *Apo*I digestion of pHSV-EYFP-RfC: calculated fragments: 4889bp, 2016bp, 370bp
Lane 3: *Blp*I digestion of pHSV-EYFP-RfC: calculated fragment: 7275bp
Lane 4: *Not*I digestion of pHSV-E6-EYFP: calculated fragments: 4855bp, 1255bp
Lane 5: *Apo*I digestion of pHSV-E6-EYFP: calculated fragments: 5740bp, 370bp
Lane 6: *Blp*I digestion of pHSV-E6-EYFP: calculated fragments: 4942bp, 1168bp
Right panel: digestion of pHSV-E6-EYFP:
M: 1kb DNA ladder; K, L: clone K, L

4.3 Quantitative PCR analysis

To characterize the state of infection, the existence of CPV3 E6 mRNA in skin lesions and in healthy skin of the affected dog was determined. Glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) mRNA was used as reference.

4.3.1 Establishment of a real time PCR for CPV3 E6

In order to facilitate measurement of CPV3 E6 and GAPDH mRNA levels, quantitative PCR-assays for CPV3 E6 and canine GAPDH DNA level were established first. Standard curves of two plasmids containing CPV3 E6 DNA (pHSV-E6-EYFP) and canine GAPDH (pTOPO-GAPDH) in a known concentration were created from a 10-fold serial dilution (from 10^6 to 10^3 copies/ reaction) and used for absolute quantification of DNA. Figure 6 shows these standardcurves with a slope of –3.295 and a correlations coefficient of 0.985 (pTOPO-GAPDH) and a slope of –4.033 and a correlations coefficient of 0.980 (pHSV-E6-EYFP). Thus, these standardcurves could be used for the measurement and the absolute quantification of cDNA obtained by reverse transcription of RNA samples.



a **b**

Figure 6: Standardcurves of pTOPO-GAPDH (a) and pHSV-E6-EYFP (b) created from a 10-fold serial dilutions from 10^6 to 10^3 copies/ reaction.

4.3.2 Detection of CPV3 E6 mRNA in several skin lesions

In order to evaluate the presence of CPV3 E6-mRNA in the skin lesions of the affected dog, RNA was extracted from three different lesions and from macroscopically normal-appearing skin and reverse transcribed. Since the E6 specific primer pair used was not able to distinguish between genomic viral DNA and reverse transcribed cDNA, mRNA-samples analysed as negative controls, were treated in exactly the same way but the reverse transcriptase was substituted by water (no-RT samples). After reverse transcription quantitative real-time PCR was carried out. The Ct values from skin samples were used in combination with the standard curves of pTOPO-GAPDH and pHSV-E6-EYFP to calculate the copy numbers of E6 and GAPDH mRNA. To avoid amplification from genomic DNA, several dilutions of cDNA extracts were analysed. The dilution, which gave no signal in the absence of reverse transcriptase was chosen for measuring E6 mRNA levels. The difference in the Ct value was about +3.3 per 1:10 dilution, which is the value of optimal doubling of DNA amount per cycle. Since the amount of used RNA sample was not standardised, we compared different sample dilutions of the cDNA extracts. Figure 7 shows the dilution of different samples, which resulted in a GAPDH mRNA content between 10^3 and 10^4 copies. Therefore, the presented dilutions are 1:10 for healthy skin and lesion 1, 1:100 for lesion 2 and 1:1000 for lesion 3a and 3b. Every sample

was analysed at least two times. Table 3 shows the calculated copy numbers of E6 and GAPDH mRNA corresponding to Figure 7. E6 cDNA measurement of the 1:1000 dilution of lesion 3b revealed a positive Ct value of the no-RT sample, whereas the no-RT sample of the 1:10.000 dilution was negative. In this case we analysed the copy number of the 1:10.000 dilution and calculated the corresponding copy number of the 1:1000 dilution. The remaining no-RT samples of the dilutions used in Figure 7 were negative. Thus CPV3 E6 mRNA expression in the skin lesions of the affected dog was demonstrated, whereas it was not possible to detect any CPV3 E6 mRNA expression in healthy skin. The amount of E6 mRNA was slightly increased compared to the amount of GAPDH mRNA in the same extract.

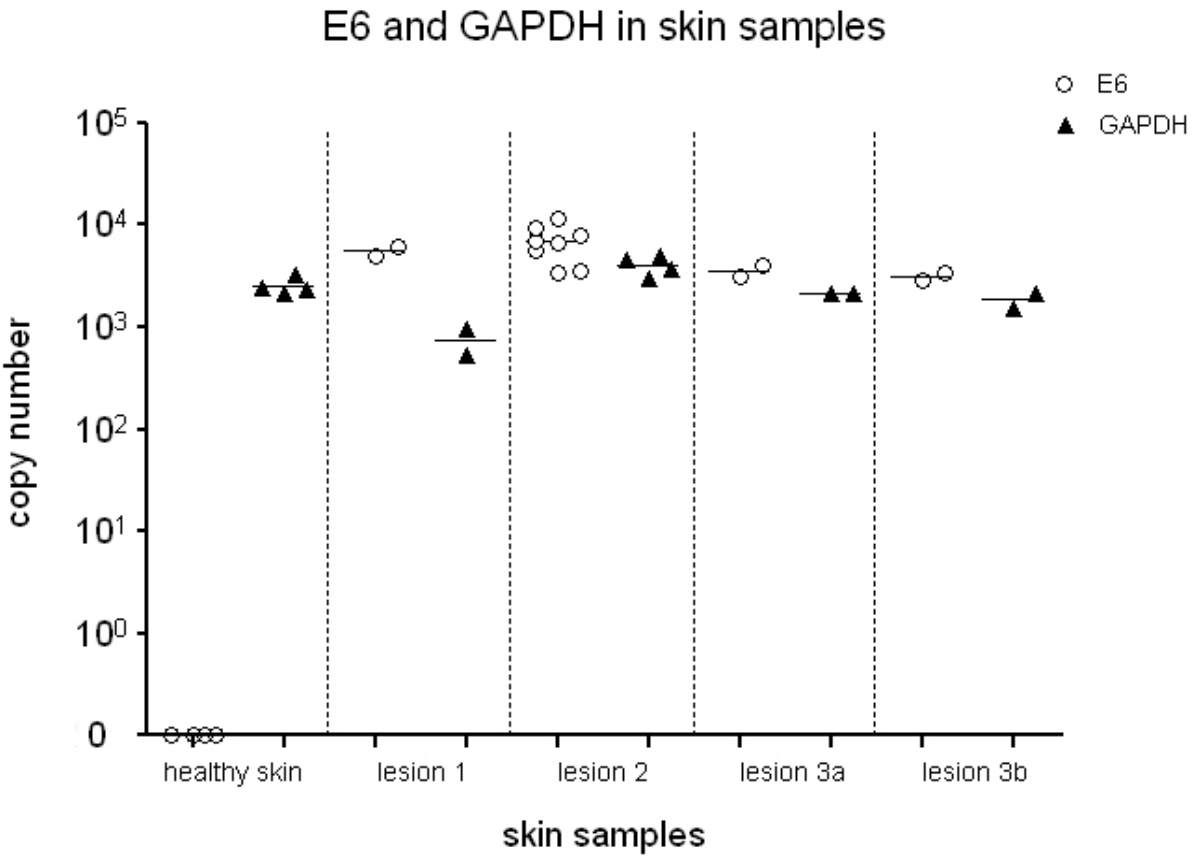


Figure 7: Results of the real time PCR: Y axis shows the copy number and X axis shows the different skin samples. Lesion 3a and 3b means it is the same lesion, but cut into two pieces. The number of circles/ triangles stands for the analysing frequency of each sample. CPV3-E6 mRNA was found in every lesion but not in healthy skin. The amount of E6 mRNA was slightly increased compared to the amount of GAPDH mRNA in the same extract. Although the amount of GAPDH of healthy skin is quite similar to the amount of GAPDH in lesions, E6 was not detectable in healthy skin.

Samples	E6	GAPDH	Samples	E6	GAPDH
Healthy skin	0	2147	Lesion 2	5572	3033
Healthy skin	0	2466	Lesion 2	7808	4587
Healthy skin	0	2302	Lesion 2	3554	3730
Healthy skin	0	3250	Lesion 2	3359	4915
Lesion 1	4980	541	Lesion 2	6596	_
Lesion 1	6236	939	Lesion 2	11574	_
Lesion 3a	3977	2149	Lesion 2	6978	_
Lesion 3a	3977	2149	Lesion 2	9243	_
Lesion 3b	2830	1522			
Lesion 3b	3350	2148			

Table 3: calculated copy number of E6 and GAPDH in the different samples. The equation of the linear regression describing the relationship between Ct-values and copy numbers of the plasmid was used for the calculation.

4.4 Characterization of CPV3 E6-fusion protein in eukaryotic cell cultures

4.4.1 Transfection of VERO cells with the pHSV-E6-EYFP amplicon vector

In order to observe the expression and compartmentalization of the CPV3 E6 gene protein product in eukaryotic cells, VERO cells were transfected with pHSV-E6-EYFP_L. The cells were observed 24 and 48 hours post transfection under a fluorescence microscope. One day post transfection about five percent of the transfected cells showed yellow fluorescence, corresponding to the E6-EYFP fusion protein. This observation indicated, that E6-EYFP protein was expressed. Fluorescence seemed to be detectable only in the nuclei of positive cells. Observation of the cells by light microscopy revealed a star-like appearance implying a healthy state of the cells 24 hours post transfection. In order to verify the localization in the nucleus, nuclei were stained with Hoechst after 48 hours. An overlay of the pictures taken with YFP filter (Figure 3a), with DAPI filter (Figure 3b) and with normal light (Figure 3c) revealed that yellow fluorescence was accumulated exclusively in the nuclei (Figure 3d). At that time the cell profile of fluorescent positive cells by normal light microscopy was round shaped implying the cell to be dying (Figure 3c).

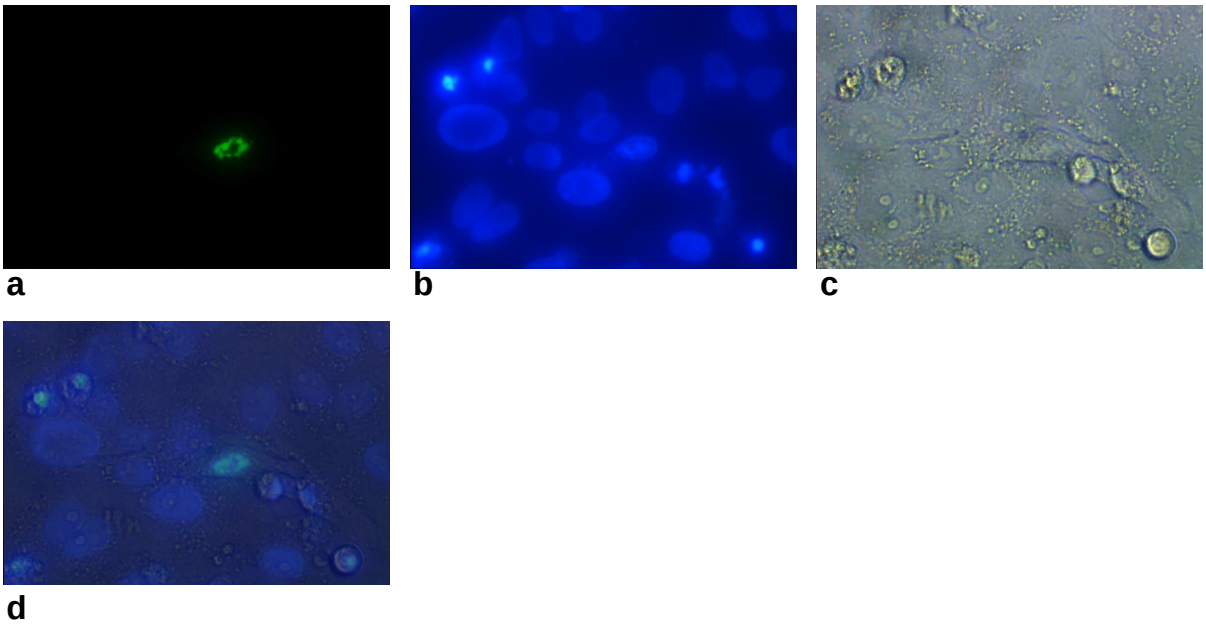


Figure 3: Vero cells 48 hours post transfection with pHSV-E6-EYFP: Cells were observed with a 40x objective in an inverse microscope using an UV filter for visualization of the fluorescence. Yellow fluorescence: E6-EYFP, blue fluorescence: nuclei stained with Hoechst. Cells were observed **a)** using the YFP filter **b)** using the DAPI filter **c)** with normal light and **d)** Overlay of pictures a, b and c: yellow fluorescence was accumulated exclusively in the nucleus of the transfected cells.

4.4.2 Transduction of VERO/ VERO 2-2 cells with pHSV-E6-EYFP amplicon particles

In order to achieve expression of the CPV3 E6 gene protein product under more gentle conditions, pHSV-E6-EYFP was packaged into HSV-1 particles. Therefore, Vero 2-2 cells were co-transfected with pHSV-E6-EYFP_L, a replication-competent, packaging-defective HSV-1 helper genome (fHSVΔpacΔ27) and an additional helper plasmid (pEBHICP27). The cells were observed 24 and 72 hours post transfection. After 24 hours about fifty percent of the transfected cells showed yellow fluorescence, corresponding to the E6-EYFP fusion protein. The amount of fluorescent cells as well as the intensity of fluorescence was higher than in absence of HSV-1, indicating that pHSV-E6-EYFP was augmented. Interestingly the amount of fluorescent cells was reduced after 72 hours. At that time amplicon particles were harvested. These amplicon particles were used to transduce VERO cells. After 24 hours cells were fixed on cover slips with 3% Paraformaldehyde and stained with DAPI. Figure 4 shows VERO cells one day post transduction with E6-amplicon particles. Again fluorescence, corresponding to the E6-EYFP protein was detected in the nucleus of the transduced cells.

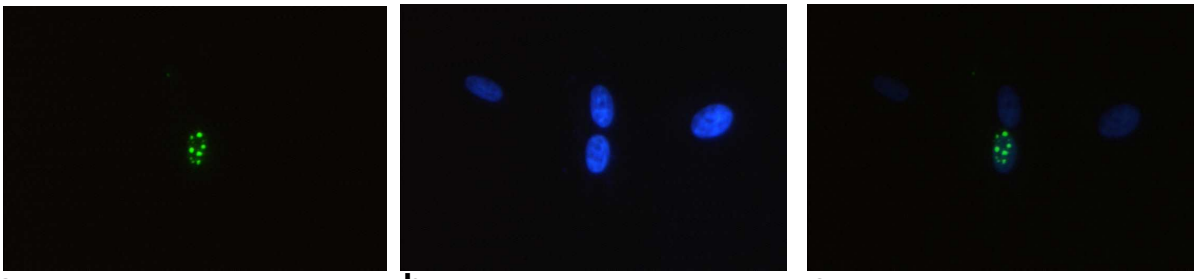


Figure 4: Vero cells 24 hours post transduction with E6-EYFP amplicon virions: Cells were observed with a 40x objective in an inverse microscope using an UV filter for visualization of the fluorescence. Yellow fluorescence: E6-EYFP, blue fluorescence: nuclei stained with Dapi. Cells were observed **a)** using the YFP filter and **b)** using the Dapi filter. **c)** Overlay of pictures a and b. Fluorescence was accumulated in the nucleus of the infected cells.

In a second experiment VERO 2-2 cells were transduced with these amplicon particles and observed 24, 48 and 72 hours post transduction. To avoid alterations of the cells with ultra violet light during observation, cells in three different wells were transduced. Observation was carried out after 24 hours in well 1, after 48 hours in well 2 and after 72 hours in well 3. As a control VERO 2-2 cells were transduced with EYFP amplicon particles which were packaged at the same time with equal conditions. One day post transduction about five percent of the transduced cells showed yellow fluorescence, corresponding to the E6-EYFP fusion protein (Figure 5a). Observation of the cells by light microscopy revealed a star-like appearance implying a healthy state of the cells (Figure 5b). Two days post transduction fluorescence agglutinated in the nucleus (Figure 5c). The cell profile of fluorescent cells by normal light microscopy was shortened implying the cell to be dying (Figure 5d). On day three post transduction fluorescent particles were swimming over the cell layer (Figure 5e). Observation of the cells by light microscopy revealed that cells corresponding to the fluorescent particles were round and detached from the monolayer cell culture implying a dead state of the fluorescent cells (Figure 5f). In contrast the cell profile of VERO 2-2 cells transduced with EYFP amplicon particles observed by light microscopy was star-like shaped on day one, two and three after transduction (Figure 5h). Thus, transduction of eukaryotic cells with pHSV-E6-EYFP amplicon particles resulted in two different observations. First, the E6-EYFP fusion protein was shown to localize exclusively in the nucleus. And second, transduction of pHSV-E6-EYFP amplicon particles resulted in the death of cells after 72h.

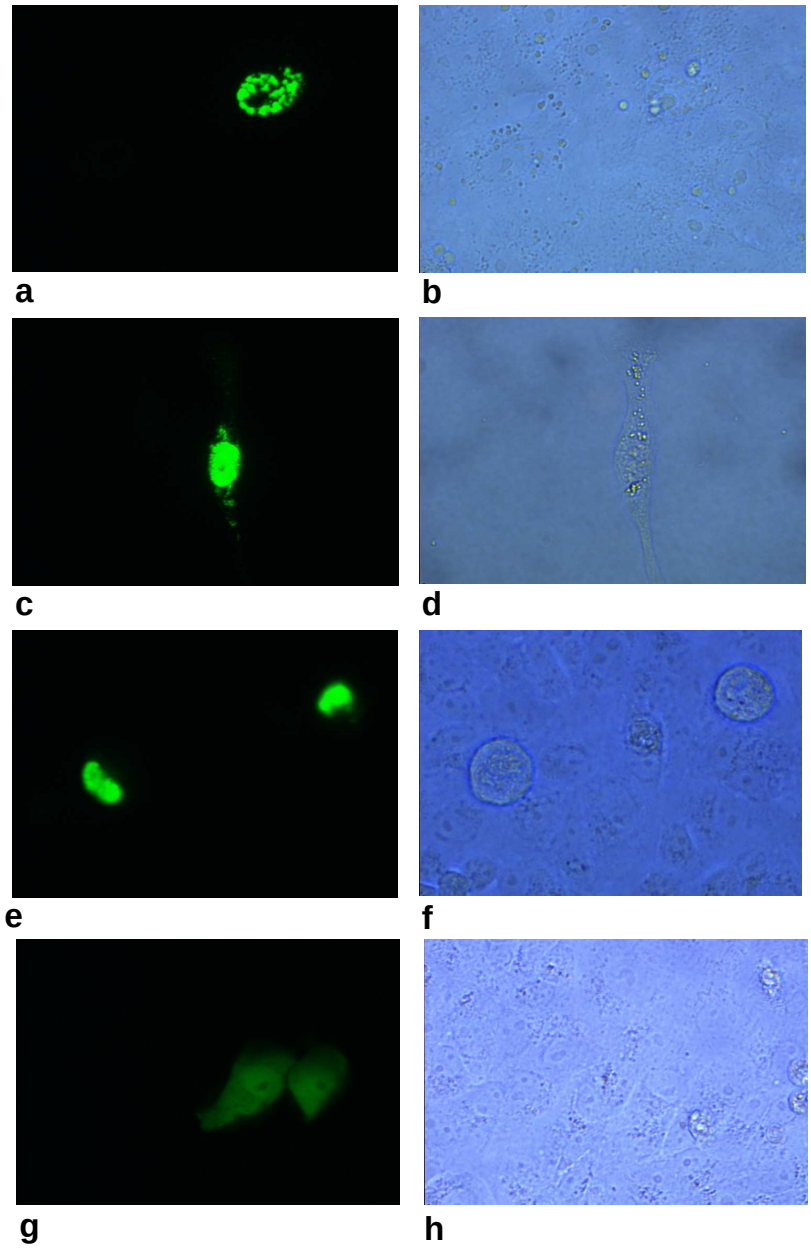


Figure 5: VERO 2-2 cells 24 (a, b), 48 (b, c) and 72 (e, f, g, h) hours post transduction with E6-EYFP amplicon virions (a-f) respectively EYFP amplicon virions (g-h): Cells were observed with a 40x objective in an inverse microscope using an YFP filter for visualization of the fluorescence. Yellow fluorescence: E6-EYFP (a, c, e) EYFP (g). Cells were observed a, c, e, g: using the YFP filter b, d, f, h: with normal light.

4.5 Expression of HSV-1 Amplicon-encoded siRNAs

4.5.1 Generation and packaging of siRNAs

In order to construct a siRNA targeting CPV3 E6 mRNA, DNA oligonucleotides targeting E6 at two different locations were synthesized as 64-mer sense and antisense oligonucleotide templates and inserted into an amplicon vector (pHSVsuper) between the

*Bgl*II and the *Hind*III sites (pHSVsiE6-2 and pHSVsiE6-3) as describrd in Materials and Methods. To identify clones containing the desired DNA oligonucleotides, twelve colonies per plasmid were picked. Plasmid DNA was isolated and restriction analysis with two restriction enzymes (*Eco*RI and *Hind*III) was performed. Six (pHSVsiE6-2) respectively eight (pHSVsiE6-3) of the twelve isolated clones showed the calculated restriction pattern. Amplicon packaging and titration was performed as described in material and methods. Titration revealed titers of 2.72 x 10^7 TU/ml for pHSVsiE6-3, 2.66 x 10^7 TU/ml for pHSVsiEGFP and 2 x 10^7 TU/ml for pHSVsuper. Packaging of pHSVsiE6-2 was not successful.

4.5.2 Conversion of the amplicon vector pHSV-E6-EYFP into pHSV-E6-mRFP

To differentiate the expression of CPV3 E6 protein from the expression of the siRNAs, it was necessary to change the fluorescent protein of pHSV-E6-EYFP. Therefore pHSV-E6-EYFP was digested with Asp718 and *Eco*RI to remove the EYFP and pcDNA-mRFP1-N was digested with the same enzymes to isolate the mRFP ORF. pHSV-E6 was ligated with the linearized mRFP. This ligation reaction was transformed into *E.coli*. Plasmid DNA was isolated from 12 colonies and restriction analysis with *Bam*HI was performed to confirm the presence of the insert. Nine of the isolated expression clones showed the calculated restriction pattern. To confirm that the EYFP was replaced by mRFP, restriction analysis of two clones showing the calculated restriction pattern (pHSV-E6-mRFP_2 and pHSV-E6-mRFP_4) with two more restriction enzymes (*Ava*I and *Mlu*I) was performed. As shown in figure 8 both clones showed the calculated restriction pattern. These results suggest that the fused fluorescent protein EYFP of pHSV-E6-EYFP had been replaced by mRFP.

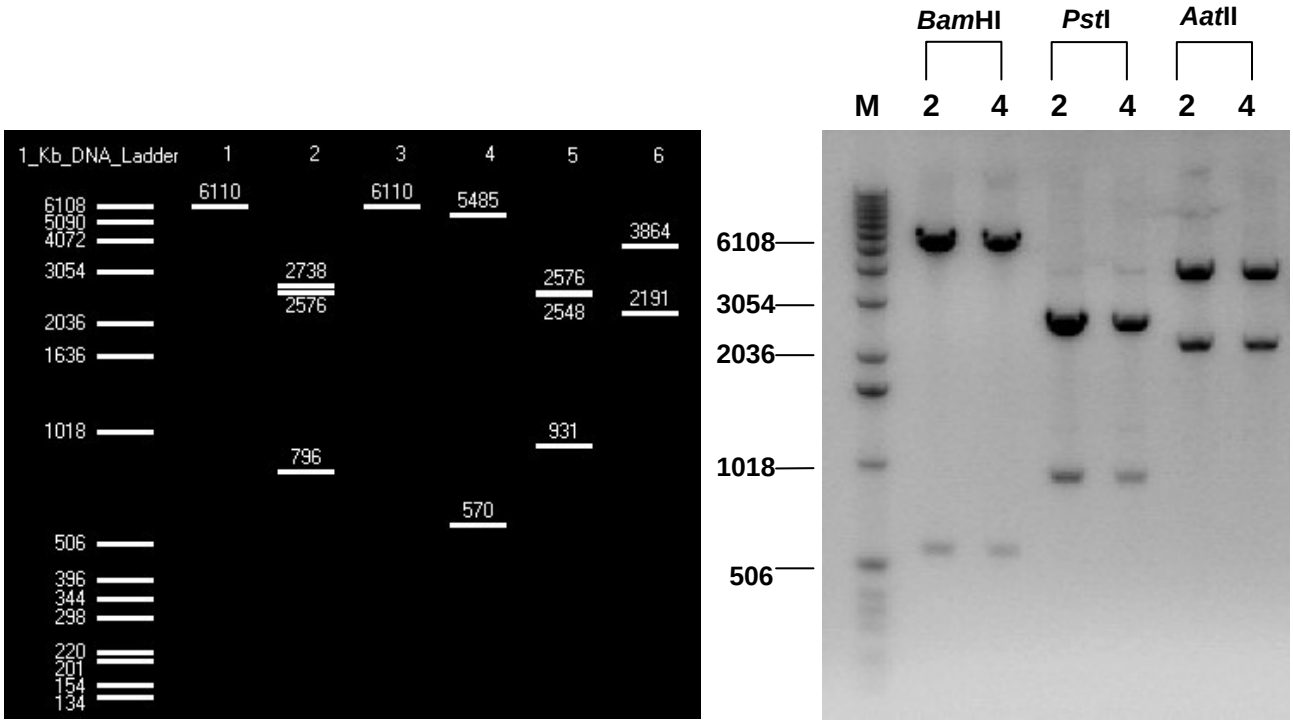


Figure 8: Restriction analysis of the amplicon expression clone pENTR-E6-mRFP on agarose gel
Left panel: virtual digestion:
Lane 1: *Bam*HI digestion of pHSV-E6-EYFP: calculated fragment: 6110bp
Lane 2: *Pst*I digestion of pHSV-E6-EYFP: calculated fragments: 2738bp, 2576bp, 796bp
Lane 3: *Aat*II digestion of pHSV-E6-EYFP: calculated fragment: 6110bp
Lane 4: *Bam*HI digestion of pHSV-E6-mRFP: calculated fragments: 5485bp, 570bp
Lane 5: *Pst*I digestion of pHSV-E6-mRFP: calculated fragments: 2576bp, 2548 bp, 931bp
Lane 6: *Aat*II digestion of pHSV-E6-mRFP: calculated fragments: 3864bp, 2191bp
Right panel: digestion of pHSV-E6-mRFP:
M: 1kb DNA ladder; 2, 4: clone 2, 4

4.5.3 Transfection of siRNA infected VERO 2-2 cells with pHSV-E6-mRFP

In order to block E6 protein expression in eukaryotic cells, VERO 2-2 cells were transduced with pHSVsiE6-3 with a multiplicity of infection (m.o.i.) of one respectively five TU per cell on day one. Amplicons that contained DNA oligonucleotides targeting EGFP (pHSVsiEGFP) and pHSVsuper were used as controls. On day two these cells were transfected with pHSV-E6-mRFP. Cells were observed on day two, three, four and five using a fluorescent microscope, which allows co-detection of both EGFP (green, amplicon-transduced cells) and mRFP-E6 (red, transfected cells). One day after transduction about 75% of the cells showed green fluorescence indicating, that the amplicon DNA had entered these cells and, thus siRNA was expressed. One day after transfection with pHSV-E6-mRFP about 5% of the cells showed red fluorescence indicating that the E6 fusion protein was expressed. About 50% of the red cells showed coexistent green

fluorescence. No cells with bright green fluorescence and coexistent red fluorescence could be detected. These observations could be made in cells transduced with pHSVsiE6-3 as well as in cells transduced with control amplicons pHSVsiEGFP and pHSVsuper (Figure 9a). Observations on day four were quite similar, whereas on day five cells expressing the red fluorescence looked round and dead while cells expressig only the green fluorescence had a star-like appearance implying a healthy state. Again this could be observed in cells transduced with pHSVsiE6-3 as well as in cells transduced with pHSVsiEGFP and pHSVsuper (Figure 9b). Thus no obvious reduction of red fluorescence (mRFP-E6) could be detected in cells treated with E6-specific siRNA (pHSVsiE6-3) compared to cells treated with control amplicons (pHSVsiEGFP and pHSVsuper).

The purpose of the experiment was to evaluate a reduction of CPV3 E6 after incubating the cells with siRNA targeting CPV3 E6 (pHSVsiE6-3). For further control a vector without siRNA (pHSVsuper) and another vector with siRNA targeting EGFP (pHSVsiEGFP) was used. As expected in cells transduced with pHSVsuper no effect was observed four days after transduction. In cells transduced with pHSVsiEGFP no reduction of green fluorescence and in cells transduced with pHSVsiE6-3 no reduction of red fluorescence were recognized four days after transduction. It is known from the literature that the effect of eliminating green fluorescence by pHSVsiEGFP was observed seven days after transduction.[45] Observation of cells seven days after transduction was not possible, because cells died three days after transfection with pHSV-E6-mRFP.

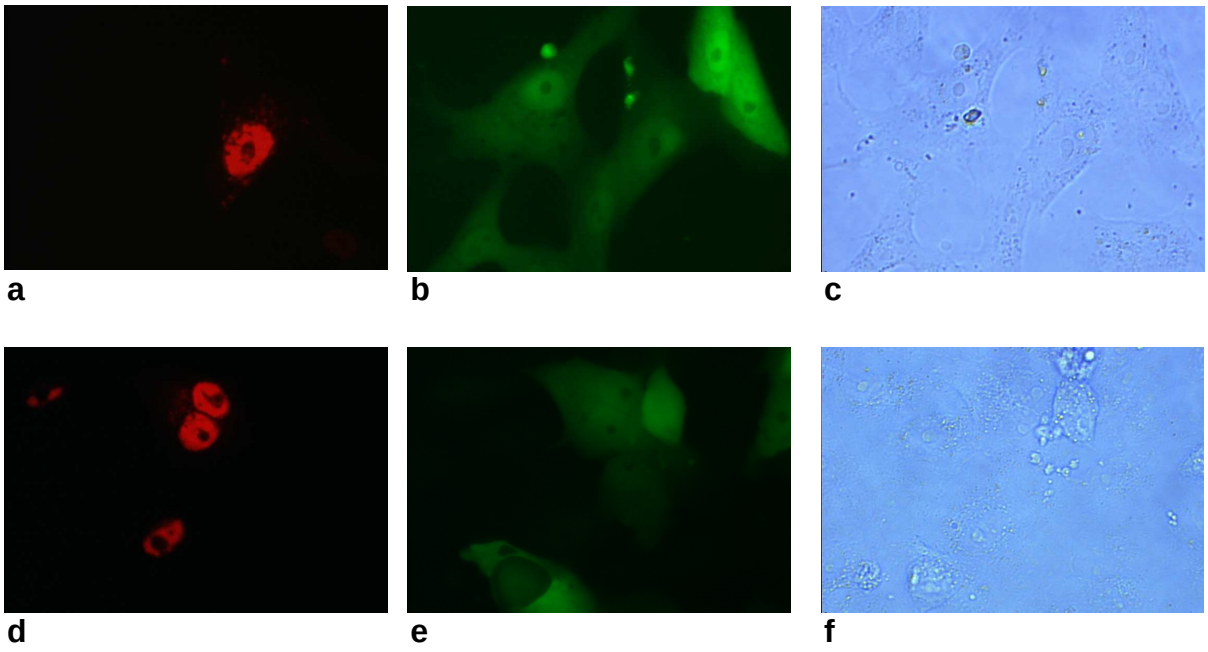


Figure 9a: VERO 2-2 cells 48 hours post transduction with pHSVsiE6-3 (a-c), pHSVsiEGFP (d-f) and 24 hours post transfection with pHSV-E6-mRFP: Cells were observed with a 40x objective in an inverse microscope using an UV filter for visualization of the fluorescence. Green fluorescence: GFP, red fluorescence: E6-mRFP. Cells were observed **a and d:** using the dsRed filter, **b and e:** using the GFP filter and **c and f:** with normal light. Red and green fluorescence in the same cell could be detected in pHSVsiE6-3 as well as in pHSVsiEGFP infected cells.

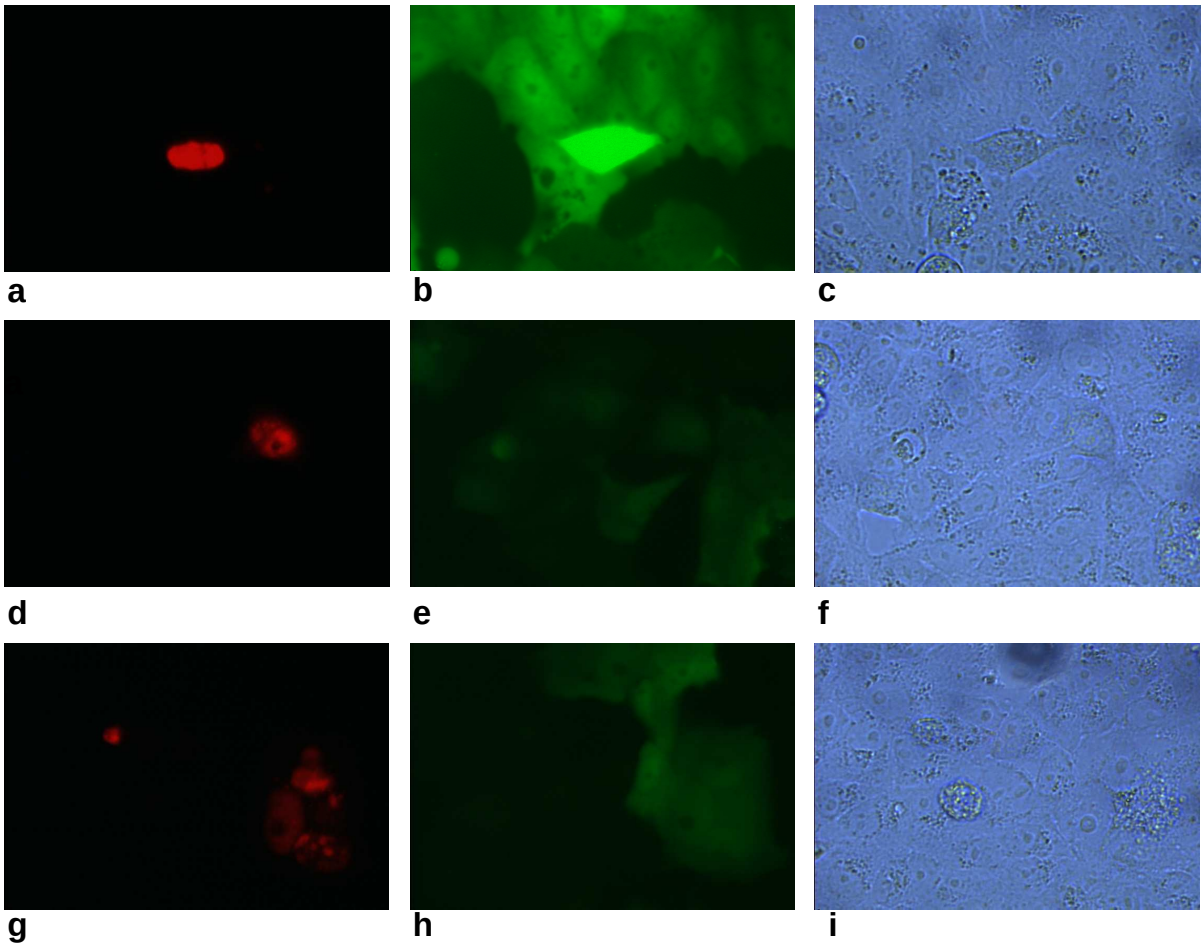


Figure 9b: VERO 2-2 cells 4 days post transduction with pHSVsiE6-3 (a-c), pHSVsiEGFP (d-f), vector without siRNA (g-i) and 3 days post transfection with pHSV-E6-mRFP: Cells were observed with a 40x objective in an inverse microscope using an UV filter for visualization of the fluorescence. Green fluorescence: GFP, red fluorescence: E6-mRFP. Cells were observed **a, d, g:** using the dsRed filter, **b, e, h:** using the GFP filter and **c, f, i:** with normal light.

5 Discussion

Recently, we reported the cloning and sequencing of a novel canine PV genome (CPV3), isolated from a SCC of a dog with EV like skin lesions.[5] The present work further analyses the role of CPV3 in the development of these lesions. In order to exclude other causative factors the p53 mRNA was sequenced and tested negative for mutations. Quantification of viral gene expression was achieved by evaluating the presence of CPV3 E6-mRNA in lesional and healthy skin. Presumably CPV3 plays an important role in the initiation of skin cancerogenesis as CPV3-E6 mRNA was detected in three different skin lesions, but not in healthy skin. To further characterize the putative transforming protein CPV3 E6 the expression and localization of the CPV3 protein in eukaryotic cells and effects of a siRNA targeting the CPV3 E6 gene on the E6 protein expression in cell cultures have been examined.

Numerous studies suggest that UVR plays an important role in skin cancerogenesis.[15,19] This seemed to be unlikely in our case, because the SCC developed interdigitally, a place which is not reached by much UVR. Nevertheless, to eliminate the possibility of an UVR induced mutation of the p53 gene we analysed the nucleotide sequence of a large fragment of the p53 cDNA, isolated from healthy and lesional skin of the affected dog. Comparing the sequence with the canine p53 sequence, published by Setoguchi et al., revealed an identical sequence.[50] Setoguchi found a DNA polymorphism in canine p53 at codon 325 (CTC or CGC). We found a G in all of the four sequenced clones at nucleotide 974, thus codon 325 represented the CGC type. In human tumors mutational hot spots have been identified in the p53 gene. They are localized at codons 175, 245, 248, 249 and 273.[51] There are several reports on mutational analyses of the p53 gene in tumors of dogs, but most of them concentrated on regions known to contain the majority of missense mutations in human tumors. Setoguchi et al. were the first, who examined the entire ORF of the p53 cDNA of various tumors in dogs and they found a higher frequency of p53 mutations, whereas only few were located at human mutational hot spots. They revealed 30 different mutation sites from codon 28 to codon 376. Therefore, it seems to be rational to analyse the entire ORF and to find specific canine p53 mutational hot spots rather than to extrapolate them from human p53 to canine p53 sequence. Our shortened sequence covered 29 of these 30 mutations sites reported by Setoguchi. The mutation site at codon 376, presenting a silent mutation, was missing. Sequencing two different clones of the p53

cDNA derived from healthy as well as from lesional skin of resulted in the same p53 sequence as published before. Hence, a mutation of the p53 gene in these skin lesions is most unlikely. Furthermore an immunsuppression of this dog due to other diseases or immunosuppressive treatment was excluded. Thus, CPV3 as a causative agent in the development of these skin lesions seemed to be evident.

Real-time PCR analysis of skin samples of the affected dog revealed the presence of CPV3 E6-mRNA in all three lesions which were subject of the assay. Although the amount of GAPDH of healthy skin was in a similar range as in lesions, healthy skin did not contain any detectable CPV3 E6-mRNA. In lesion 1, it was even possible to demonstrate E6 mRNA, although this sample contained less GAPDH mRNA compared to healthy skin (Figure 7). The detection of E6 mRNA exclusively in lesional skin indicated a relationship between CPV3 in the development of skin lesions. But whether or not CPV3 was able to induce cancer transformation and whether the virus was indeed the causative agent for the lesions remained not proven.

The E6 gene was heterologously expressed by transfection of plasmids and transduction of amplicons. Observation of the intracellular localization of the CPV E6 protein showed an accumulation in dot-like structures within the nucleus. Guccione et al. reported differences in the cellular localization of low- and high-risk HPV E6 proteins. They found, the low risk HPV-11 E6 protein to be localized in the nucleus, whereas the high risk HPV-16 E6 protein was distributed throughout the cell. Furthermore they demonstrated that HPV-11 E6 proteins accumulated in promyelocytic leukaemia (PML) bodies, also known as the PML oncogenic domains (PODs) but the HPV-16 E6 proteins did not.[52] The Transcription of the E6E7 cassette of high risk HPVs yields full-length mRNA as well as two spliced products: E6*IE7 and E6*IIE7. Vaeteewoottacharn et al. determined the cellular distribution of the HPV 16 E6 wildtype protein, the E6*IE7 protein and an E6 splice donor mutant (E6MT) protein. They concluded, that the wild type E6 construct, which yields both full-length E6 and E6*I proteins, and the E6*I construct were expressed throughout the cells, whereas the E6MT construct was primarily expressed in the nucleus.[53] Taking these results into consideration with our observation that CPV3 E6 is exclusively expressed in the nucleus, it could be presumed, that the CPV3 E6 gene is not spliced and resemble therefore the HPV low-risk E6 proteins. In contrast, Tao et al. demonstrated that high-risk full-length E6 proteins are distributed predominantly in the nucleus, whereas low-risk full-length E6 proteins are localized in the cytoplasm.[54] However, varying protein concentrations could

explain different protein distributions throughout the cell. Thus it is difficult to predict an oncogenic potential of a PV just because of the localization of its E6 protein.

Expression of CPV3 E6 protein in eukaryotic cells resulted in a cell shortening and round appearance implying apoptosis. Thus transfection as well as transduction of CPV3 E6 gene resulted in cell death. This result was in contrast to the expected function of the E6 protein, which was predicted to prevent apoptosis. A possible explanation for this observation would be that immortalization can only be obtained by a joint function of E6 and E7. Zur Hausen reviewed that indeed immortalization of various human cells can be achieved with either E6 or E7 genes of high risk HPVs and even more efficiently by their combined function.[35] Several reports support the concept, that neither the individual genes E6 and E7 nor their cooperation is sufficient to convert normal cells into an immortalized or malignant state.[35] However, it is yet unexplained why the introduction of CPV3 E6 resulted in cell death. An explanation would be either CPV3 E6 itself had detrimental properties or another condition, for example the fusion to EYFP, resulted in a noxious effect. In transfected cells, the act of transfection would possibly be noxious and cause cell death.

Transduction with purified amplicon particles revealed no protein expression. A detrimental biological activity of the CPV3 E6 protein either destroying the cells themselves or interfering with the HSV-1 function could explain this finding. Thus non-purified amplicon particles with much “foreign material” (consisting of cell debris) were used to transduce cells with E6. This amount of “foreign material” could be responsible for the resulting cell death. But in contrast transduction with control EYFP amplicon particles, which were treated in exactly the same way revealed not the same cell pattern. Thus a putative noxious action of the protein itself can be supposed. This potentially damaging effect of CPV3 E6 could also be responsible for the relatively low titers resulting of packaging of this gene. However, further studies are necessary to either confirm or disprove these interpretations.

Another approach would be to combine the findings of E6 accumulation in dot-like structures within the nucleus and cells death. If the dot-like structures represent PODs and the cell dies of apoptosis, the question about a connection between these conditions arise. Although the molecular function of PODs is currently not clear, there is accumulating evidence that they represent regulatory domains involved in various biological processes, including amongst others an apoptotic process.[55] Guo et al. reported about an interaction and co-localization of the PML and the p53 protein in PODs and defined a PML-dependent, p53-regulatory pathway for apoptosis.[56] There are two principle apoptotic

pathways, (1) the intrinsic, mitochondria regulated pathway and (2) the extrinsic pathway, divided in a receptor-induced pathway and a nuclear apoptotic pathway. At this point of the study it would go to far to predict an apoptotic pathway, possibly affected by CPV3 E6. There are few arguments for the nuclear apoptotic pathway, maybe due to regulation of p53 ubiquitination. Further studies are necessary to determine, whether CPV3 E6 can interfere with an apoptotic pathway and if yes with which one.

Treatment of cells with a siRNA targeting CPV3 E6 did not result in decreased expression of the E6 protein. We suggest several possible interpretations for this observation. First and foremost the use of a single siRNA targeting only one sequence of the CPV3 E6 gene is probably not sufficient. Reynolds et al. investigated the performance and the widely varying silencing abilities of 180 siRNAs targeting the mRNA of two genes.[57] We designed a siRNA which targets the coding region of E6 predicted on the base of multiple sequence alignments to various PV E6 amino acid sequences. However, we cannot exclude that a sequence was targeted, whose blocking did not result in the desired effect. A broader approach for targeting several sites on the CDS of E6 was not possible due to time limitations. Three main concerns have to be taken into account for the consideration of our results. First, the fusion of E6 to mRFP resulted in a detrimental effect. Second, the amount of siRNA one day after transduction was not sufficient to inhibit the protein expression. And finally, due to transfection, the targeting mRNA existed in abundance and therefore difficult for the siRNA to work effectively. However, Saydam et al. reported the successful use of a siRNA under similar experimental conditions, in an experiment involving transduction of the siRNA 4 hours after transfection with the targeted gene.[58]

Outlook: To define the role of CPV3 with regard to the aforementioned skin lesions, more details need to be known about the transforming properties not only of CPV3 E6, but also of CPV3 E7 and a combination of these two proteins. Therefore, further characterisation the CPV3 E7 protein and the effects of both putative transforming proteins E6 and E7 are necessary. To verify the hypothesis, that the CPV3 E6 protein induces cell death, it would be necessary to exclude the influence of the fluorescent fusion partner (EYFP or mRFP). Therefore, following approaches are necessary: 1) analysis of E6, which is not fused to EYFP or mRFP 2) analysis of E6, genetically fused to EYFP or mRFP sequences, but with the fusion partner in a separate reading frame to avoid its translation and 3) visualization of E6 by antibodies, either by anti-E6 antibodies or by taking advantage of a very small SV5-derived tag, against which antibodies are commercially available. To better imitate the

natural host the use of canine kerationcytes cultures would be advantageous. In our RT-PCR study it was difficult to fully eliminate the genomic DNA of CPV3. Thus it would be interesting to investigate the splicing pattern of CPV3 and construct mRNA specific primers facilitating the quantification of mRNA content in cell cultures. Finally, testing various siRNAs targeting CPV3 E6 and E7 in CPV3-positive cell lines, derived from a lesion of the affected dog would be of great interest, particularly to develop a causative therapy for this dog.

6 References

(1) **Hengge** UR, Esser S, Schultewolter T, Behrendt C, Meyer T, Stockfleth E, Goos M. Self-administered topical 5% imiquimod for the treatment of common warts and molluscum contagiosum. Br J Dermatol 2000; 143:1026-1031.

(2) **Hengge** UR, Schaller J. Successful treatment of invasive squamous cell carcinoma using topical Imiquimod. Arch Dermatol 2004; 140:404-406.

(3) **Zaugg** N, Nespeca G, Hauser B, Ackermann M, Favrot C. Detection of novel papillomaviruses in canine mucosal, cutaneous and in situ squamous cell carcinomas. Vet. Dermatology 2005; 16:290-298.

(4) **Rector** A, Tachezy R, Van Ranst M. A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. J. Virol. 2004; 78:4993-4998.

(5) **Tobler** K, Favrot C, Nespeca G, Ackermann M. Detection of the prototype of a potential novel genus among the papillomaviridae in association with canine epidemodysplasia verruciformis. Manuscript in preperation.

(6) reviewed in **Saladi** RN, Persaud AN. The causes of skin cancer: a comprehensive review. Drugs Today 2005; 41(1):37-53.

(7) **Majewski** S, Jablonska S. Epidermodysplasia verruciformis as a model of human papillomavirus-induced genetic cancer of the skin. Arch Dermatol. 1995; 131:1312-1318.

(8) **Jablonska** S, Dabrowski J, Jakubowicz K. Epidermodysplasia verruciformis as a model in studies on the role of papovaviruses in oncogenesis. Cancer Res 1972; 32(3):583-589.

(9) **Berkhout** RJ, Tieben LM, Smits HL, Bavinck JN, Vermeer BJ, ter Schegget J. Nested PCR approach for detection and typing of epidermodysplasia verruciformis-associated human papillomavirus types in cutaneous cancers from renal transplant recipients. J Clin. Microbiol 1995; 33:690-695.

(10) **Lindelöf** B, Sigurgeirsson B, Gäbel H, Stern RS. Incidence of skin cancer in 5356 patients following organ transplantation. Br J Dermatol 2000; 143:513-519.

(11) **Antonsson** A, Forslund O, Ekberg H, Sterner G, Hansson BG. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J Virol 2000; 74(24):11636-11641.

(12) **Boxman** IL, Berkhout RJ, Mulder LH, Wolkers MC, Bouwes Bavinck JN, Vermeer BJ, ter Schegget J. Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. J Invst Dermatol 1997; 108(5):712-715.

(13) **Favre** M, Orth G, Majewski S, Baloul S, Pura A, Jablonska S. Psoriasis, a possible reservoir for human papillomavirus type 5, the virus associated with skin carcinomas of Epidermodysplasia verruciformis. J Invst Dermatol 1998; 110(4):311-317.

(14) **Weissenborn** SJ, Höpfl R, Weber F, Smola H, Pfister HJ, Fuchs PG. High prevalence of a variety of epidermodysplasia verruciformis-associated human papillomaviruses in psoriatic skin of patients treated or not treated with PUVA. J Invst Dermatol 1999; 113(1):122-126.

(15) reviewed in **Alam** M, Ratner D. Cutaneous squamous-cell carcinoma. N Engl J Med 2001; 344(13):975-983.

(16) **Jackson** S, Storey A. E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage. Oncogene 2000; 19(4):592-598.

(17) **Padlewska** K, Ramoz N, Cassonet P, Riou G, Barrois M, Majewski S, Croissant O, Jablonska S, Orth G. Mutation and abnormal expression of the p53 gene in the viral skin carcinogenesis of Epidermodysplasia verruciformis. J Invst Dermatol 2001; 117:935-942.

(18) **Caldeira** S, Filotico R, Accardi R, Zehbe I, Franceschi S, Tommasino M. p53 mutations are common in human papillomavirus type 38-positive non-melanoma skin cancers. Cancer Letters 2004; 209:119-124.

(19) reviewed in **Brash** DE. Sunligth and the onset of skin cancer. Trends Genet 1997; 13(10):410-414.

(20) **Briggs** O. Lentiginosis profunda in the pug: three case reports. J. of Small Animal Practice 1985; 26:675-680.

(21) **Waldner** E. Pigmented epidermal nevus. In Gross T, Ihrke P, Waldner E. Veterinary dermatopathology. A macroscopic and microscopic evaluation of canine and feline skin disease. St Louis: Mosby-Year Book Inc. 1992; 331-332.

(22) **Nagata** M, Nanko H, Moriyama A, Washizu T, Ishida T. Pigmented plaques associated with papillomavirus infection in dogs: Is this Epidermodysplasia verruciformis? Vet Derm 1995; 6:179-186.

(23) **Narama** I, Kobayashi Y, Yamagami T, Ozaki K, Ueda Y. Pigmented cutaneous papillomatosis (pigmented epidermal nevus) in three pug dogs; histopathology, electron microscopy and analysis of viral DNA by the polymerase chain reaction. J. Comp. Path 2005; 132:132-138.

(24) **Callan** MB, Preziosi D, Mauldin E. Multiple papillomavirus-associated epidermal harmatomas and squamous cell carcinomas in situ in a dog following chronic treatment with prednisone and cyclosporine. Vet Dermatol. 2005; 16:338-345.

(25) **Stokking** LB, Ehrhart EJ, Lichtensteiger CA, Campbell KL. Pigmented epidermal plaques in three dogs. J Am Anim Hosp Assoc. 2004; 40(5):411-417.

(26) **Tanabe** C, Kano R, Nagata M, Nakamura Y, Watanabe S, Hasegawa A. Molecular characteristics of cutaneous papillomavirus from the canine pigmented epidermal nevus. J. Vet. Med. Sci. 2000; 62(11):1189-1192.

(27) reviewed in **Latonen** L, Laiho M. Cellular UV damage responses – Functions of tumor suppressor p53. Biochim Biophys Acta 2005; 1755(2):71-89.

(28) reviewed in **Appella** E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem. 2001; 268(10):2764-2772.

(29) reviewed in **Prives** C, Hall PA. The p53 pathway. J Pathol. 1999; 187(1):112-126.

(30) **Howley** PM, Lowy DR. Papillomaviruses and their replication. In: Fields Virology, 4th edition. Philadelphia: Lippincott Williams & Wilkins, 2001; 2197-2229.

(31) reviewed in **Doorbar** J. The papillomavirus life cycle. J of Clin Virology 2005; 7-15.

(32) **De Villiers** EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology 2004; 324:17-27.

(33) **Lorincz** AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ. Human papillomavirus infection of the cervix: relative risk association of 15 common anogenital types. Obstet Gynecol 1992; 79(3):328-337.

(34) **Boxman** IL, Mulder LH, Noya F, de Waard V, Gibbs S, Broker TR, ten Kate F, Chow LT, ter Schegget J. Transduction of the E6 and E7 genes of epidermodysplasia-verruciformis-associated human papillomaviruses alters human keratinocyte growth and differentiation in organotypic cultures. J Invest Dermatol 2001; 117(6):1397-1404.

(35) reviewed in **Zur Hausen** H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000; 92(9):690-698.

(36) **Watanabe** S, Kanda T, Yoshiike K. Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. J Virol1989; 63(2):965-969.

(37) **Scheffner** M, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquination of p53. Cell 1993; 75(3):495-505.

(38) **Amundson** SA, Myers TG, Fornace AJ. Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. Oncogene 1998; 17(25):3287-3299.

(39) **Dyson** N, Howley PM, Münger K, Harlow E. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 1989; 243:934-937.

(40) **Steger** G, Pfister H. In vitro expressed HPV 8 E6 protein does not bind p53. Arch Virol 1992; 125:355-360.

(41) **Kiyono** T, Hiraiwa A, Ishii S, Takahashi T, Ishibashi M. Inhibition of p53-mediated transactivation by E6 of type 1, but not type 5, 8, or 47, human papillomavirus of cutaneous origin. J Virol 1994; 68:4656-4661.

(42) **Elbel** M, Carl S, Sparderna S, Iftner T. A comparative analysis of the interactions of the E6 proteins from cutaneous and genital papillomaviruses with p53 and E6AP in correlation to their transforming potential. Virology 1997; 239:132-149.

(43) **Jackson** S, Harwood C, Thomas M, Banks L, Storey A. Role of Bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteins. Genes Devel 2000; 14:3065-3073.

(44) reviewed in **Oehmig** A, Fraefel C, Breakefield XO. Uptate on Herpesvirus Amplicon Vectors. Mol Ther. 2004; 10:630-643.

(45) reviewed in **Hannon** GJ. RNA interference. Nature 2002; 418:244-251.

(46) **Lipardi** C, Wei Q, Paterson BM. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell 2001; 107:297-307.

(47) **Elbashir** SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001; 411:494-498.

(48) **Harborth** J, Elbashir SM, Bechert K, Tuschl T, Weber K. Identification of essential genes in cultured mammalian cells using small interfering RNAs. J Cell Sci 2001; 114:4557-4565.

(49) **Butz** K, Ristriani T, Hengstermann A, Denk C, Scheffner M, Hoppe-Seyler F. siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells. Oncogene 2003; 22:5938-5945.

(50) **Setoguchi** A, Sakai T, Okuda M, Minehata K, Yazawa M, Ishizaka T, Watari T, Nishimura R, Sasaki N, Hasegawa A, Tsujimoto H. Aberrations of the p53 tumor suppressor gene in various tumors in dogs. Am J Vet Res. 2001; 62(3):433-439.

(51) **Hollstein** M, Shomer B, Greenblatt M, Soussi T, Hovig E, Montesano R, Harris CC. Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. Nucleic Acids Res. 1996; 24(1):141-146.

(52) **Guccione** E, Massimi P, Bernat A, Banks L. Comparative analysis of the intracellular location of the high- and low-risk human papillomavirus oncoproteins. Virology 2002; 293(1):20-25.

(53) **Vaeteewoottacharn** K, Chamutpong S, Ponglikitmongkol M, Angeletti PC. Differential localization of HPV16 E6 splice products with E6-associated protein. Virol J. 2005; 2:50.

(54) **Tao** M, Kruhlak M, Xia S, Androphy E, Zheng ZM. Signals that dictate nuclear localization of human papillomavirus type 16 oncoprotein E6 in living cells. J Virol. 2003; 77(24):13232-13247.

(55) reviewed in **Hofmann** TG, Will H. Body language: the function of PML nuclear bodies in apoptosis regulation. Cell Death Differ. 2003; 10(12):1290-1299.

(56) **Guo** A, Salomoni P, Luo J, Shih A, Zhong S, Gu W, Pandolfi PP. The function of PML in p53-dependent apoptosis. Nat Cell Biol. 2000; 2(10):730-736.

(57) **Reynolds** A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol. 2004; 22(3):326-330.

(58) **Saydam** O, Glauser DL, Heid I, Turkeri G, Hilbe M, Jacobs AH, Ackermann M, Fraefel C. Herpes simplex virus 1 amplicon vector-mediated siRNA targeting epidermal growth factor receptor inhibits growth of human glioma cells in vivo. Mol Ther. 2005; 12(5):803-812.

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